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Dated: May 26, 2009 Signature: David L. Parker

Docket No.: GLOF:007USC1
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Gong et al.

Serial No.: 10/605,708

Filed: October 21, 2003

For: CHIMERIC GENE CONSTRUCTS FOR GENERATION OF FLUORESCENT TRANSGENIC ORNAMENTAL FISH

Group Art Unit: 1632

Examiner: Singh, Anoop Kumar

Atty. Dkt. No.: GLOF:007USC1

Confirmation No: 2707

APPEAL BRIEF

Commissioner for Patents
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Alexandria, VA 22313-1450

Appellants hereby submit this Appeal Brief to the Board of Patent Appeals and Interferences pursuant to 37 C.F.R. §41.31(a)(1) and 41.37 in light of the Final Office Action dated January 29, 2009 and the Notice of Appeal filed on March 27, 2009.

It is believed that the filing of the present Appeal Brief is timely, and the appropriate fees submitted. However, if any fees are due for any reason relating to the enclosed materials, the Commissioner is authorized to deduct said fees from Fulbright & Jaworski L.L.P. Account No.: 50-1212/GLOF:007USC1.

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I. REAL PARTY IN INTEREST

The real party in interest is the assignee, National University of Singapore. The subject matter of the application is currently licensed to Yorktown Technologies, L.P.

II. RELATED APPEALS, INTERFERENCES, AND JUDICIAL PROCEEDINGS

There are no related appeals, interferences or judicial proceedings that are related to, directly affect, or would be directly affected by, or have a bearing on the Board's decision in this appeal.

III. STATUS OF CLAIMS

Claims 1-3, 9-15, 20-21, 24, 30-32, 35-44 and 45 are currently pending and under rejection; Appellants hereby appeal each rejection of all of these claims.

IV. STATUS OF AMENDMENTS

There are no pending amendments.

V. SUMMARY OF CLAIMED SUBJECT MATTER

The main claim subject to rejection is claim 43, along with the support in the specification for each element (we note that the present application was electronically filed using early versions of the PTO electronic filing software, the printout of which had only page numbers and paragraph numbers), as follows:

43. A method of providing transgenic fish to the ornamental fish market *[page 8, paragraph 0013; page 39, paragraph 0091]*, comprising the steps of:

(a) obtaining a transgenic fish line comprising one or more chimeric genes that are positioned under the control of a promoter that drives the expression of a fluorescent protein in muscles of said fish *[page 6, paragraphs 0009-0010; pages 13-14, paragraphs 0028-0029; pages 18-19, paragraphs 0040-0042]*, said promoter being a muscle specific promoter *[Id.]*, such that said transgenic fish expresses fluorescent protein encoded by the gene in skeletal muscle at a level sufficient such that said transgenic fish fluoresces upon exposure to one or more light *[pages 12-13, paragraphs 0022-0026; page 35-38, paragraphs 0080-0088]*; and

(b) distributing fish of said line to the ornamental fish market *[page 1, paragraph 0002; page 6, paragraph 0010; page 39-40, paragraphs 0091-0092]*.

As can be seen, principal claim 43 does not require that the transgenic fish be capable of expressing at a high enough level to be visible in sunlight.

Turning to dependent claim 1, which depends from claim 43, it is noted that dependent claim 1 incorporates all of the limitations of claim 43, but further specifies that the selected transgenic line is capable of fluorescing upon exposure to sunlight *[see pages 37-38, paragraph 0085]*.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

A. Whether claims 1-3, 9-15, 20-21, 24, 30-32, 35-44 and 45 are properly rejected under 35 U.S.C. §112, first paragraph (non-enablement);

B. Whether claims 1-3, 9-15, 20-21, 24, 30-32, 35-44 and 45 are properly rejected under 35 U.S.C. §112, first paragraph (lack of written description); and

C. Whether claims 1-3, 9-15, 20-21, 24, 30-32, 35-44 and 45 are properly rejected on the basis of obviousness-type double patenting of US 7,135,613.

VII. ARGUMENT

A. Whether claims 1-3, 9-15, 20-21, 24, 30-32, 35-44 and 45 are properly rejected under 35 U.S.C. §112, first paragraph (non-enablement)

The Final Rejection first rejects all of the pending claims on the basis of non-enablement. Of some importance is the fact that the Action concedes that essentially the same claim as claim 1, if limited to a particular promoter (zebrafish myosin light chain gene promoter), would be fully enabled. See Action at pages 2-3. Therefore, the only enablement issue is whether the specification, in view of the level of skill in the art at the time of filing, is enabling for the use of the well-known class of muscle specific promoters in general.

We would also note that the Final Action appears to treat the main claim, claim 43, as if it requires that the transgenic fish be capable of fluorescing in sunlight. This is not the case and no such requirement appears in the main claim – the main claim 43 simply states "at a level sufficient such that said transgenic fish fluoresces upon exposure to one or more light" – and the "light" can be, for example, blue light, fluorescent light, *etc*. Thus, Appellants remarks will initially address the rejections with respect to claim 43 and then turn to a consideration of dependent claim 1. Since claim 43 merely refers to selecting transgenic fish that expresses a fluorescent protein "at a level sufficient such that said transgenic fish fluoresces upon exposure to one or more light", the issue for the Board with respect to claim 43 is merely whether the specification is enabling for the preparation of fish that have a detectable level of fluorescence when exposed to some kind of light.

Appellants would first note that the specification provides a complete description of two different muscle specific promoters that have been successfully employed in the practice of the claimed invention, including the muscle creatine kinase ("MCK") promoter and the myosin light chain ("MLC") promoter. Both of these muscle promoters were found to produce fluorescent transgenic fish ("fry") that express their fluorescence under light, in accordance with claim 43. See pages 35-38, paragraphs [0070] – [000088], particularly paragraphs [0082], [0084] and [0085], and Figs 8-12.

With respect to muscle promoters in general, which are admittedly well known in the art, Appellants refer to the Declaration of one of the inventors, Dr. Zhiyuan Gong (copy enclosed as Exhibit 1). Dr. Gong addressed a number of items in his Declaration relevant to the present appeal. For one, Dr. Gong addresses the question of the use of other muscle promoters to produce transgenic fish that express fluorescence that is visibly detectable, and explains generally why the generation of such transgenic fish, even when employing a relatively weak promoter, does not require an undue amount of experimentation. Moreover, Dr. Gong further provides evidence of the foregoing in the form of various scientific publications that have been successfully used subsequent to our priority date to achieve very high level expression of fluorescence genes in fish.

Dr. Gong starts off in paragraph 6 by noting that based on his knowledge and experience in the production of fluorescent, transgenic fish, essentially any muscle-specific promoter can be employed to produce very highly fluorescent founder embryos and lines. He continues by noting that while it may be necessary in some instances to use a screening procedure (such as the one spelled out in claim 43) to select those embryos that have appropriate position effects, this would

require only routine, repetitive steps. Dr. Gong further indicates that, of course, when a weaker promoter is employed it may be necessary to inject and screen larger numbers of embryos, which may be more than one thousand, to identify a "high expresser" but again such screening is straightforward and does not involve any additional inventiveness to accomplish. Dr. Gong concludes in paragraph 6 by stating that considering the muscle occupies a large part of the fish body and thus has the capacity to synthesize enough proteins for visible fluorescence, screening for visible fluorescence using any muscle-specific promoter provides specific guidance and predictable results for obtaining stable transgenic fish suitable for ornamental fish market.

Thus, Dr. Gong is testifying that the ability to obtain highly expressing fish is not ultimately *dependent* on the promoter. By using a highly expressing promoter, fewer embryos/fry will need to be screened as compared to using a poorer promoter, which may require that a much larger number of embryos/fry will have to be screened. However, as Dr. Gong points out, the screening method taught by his specification (and embraced by claim 43) is, in its practice, a routine procedure. See MPEP §2164; *In re Certain Limited-Charge Cell Culture Microcarriers*, 221 USPQ 1165, 1174 (Int'l Trade Comm'n 1983), *aff'd. sub nom., Massachusetts Institute of Technology v. A.B. Fortia*, 774 F.2d 1104, 227 USPQ 428 (Fed. Cir. 1985) – all standing for the proposition that the fact that experimentation may be complex does not make it "undue' where such experimentation is, as here, routine.

In paragraph 7 of his declaration, Dr. Gong provides specific examples of others that have prepared fluorescent, transgenic fish in accordance with the claimed invention, first directing the reader to the attached article of Kinoshita entitled "Transgenic medaka with brilliant fluorescence in skeletal muscle under normal light" (*Fisheries Science, 70*:645-649,

2004; attached as exhibit to Gong declaration). Dr Gong notes that, as the title implies, this article describes the preparation of transgenic, fluorescent medaka having a brilliant fluorescence in skeletal muscle under normal light. According to Dr. Gong, in these studies the author employed the skeletal muscle actin promoter, and we would further note that this author employed a promoter and enhancer construct known by others in the art at about the same time as the filing of the present application (see page 645, col. 2, reference 17). The author concludes that "[o]range, as well as green and red fluorescence was discernable with the naked eye under both daylight and fluorescent light." Page 646, col. 2 at bottom.

Dr. Gong then refers to page 648, col. 1, and observes that the author also mentions the article of Chou *et al.* (*Transgenic Res., 10*: 303-315, 2001), which is said to teach fluorescent, transgenic medaka strains with the GFP gene under the control of the β-actin gene regulatory region, which fluorescence could be observed under natural lighting (see, e.g., Figure 6, page 312). We would further note that these authors also employed gene constructs that were known in the art prior to the filing of the present application (see page 304 – to top of page 305; section entitled "Plasmids").

The foregoing observations set out in paragraph 7 of the Gong declaration are also of particular relevance to dependent claim 1, which specifies transgenic fluorescent fish that express a sunlight-visible fluorescence, in that both Chou and Kinoshita employ gene constructs that were known prior to Appellants filing date and using techniques similar to those outlined by the present inventors in the subject specification.

The Action is essentially devoid of any cognizable evidence that would support a *prima facie* conclusion of non-enablement of claim 43. The Action, at the top of page 5, refers to the

articles of Moss *et al.*, Higashijima *et al.*, Kuo *et al.*, Kim *et al.* and Hackett *et al.* (attached as Exhibits 2-6, respectively), and states that these references show "that in spite of these constructs being reproducibly expressed in a tissue specific manner, none of these promoters were suitable for use in generating transgenic ornamental fish because an unusually high level of expression is required in the muscle tissue to be of commercial value." Thus, the Action concedes that the many and various muscle specific promoters are both reproducible and specific, the argument turns entirely on their "commercial value." Such, of course, is a merely a matter of taste not an enablement issue – it is true that the licensee of the present invention, known commercially as "GloFish" (see www.glofish.com), has had much commercial success with its transgenic ornamental fish precisely because other potentially competing fish (imported from Asia) do not, in our estimation, exhibit a sunlight-visible fluorescence. Nonetheless, those fish are also being marketed commercially. Stated another way, what the Examiner is inappropriately relying on as evidence of "lack of commercial appeal" as evidence of "non-enablement." Clearly, commercial acceptance or appeal is not relevant to enablement or operability. *Phillips Petroleum Co. v. U.S. Steel Corp.*, 673 F.Supp. 1278, 6 USPQ2d 1065, 1104-05 (D. Del. 1987), *aff'd*, 865 F.2d 1247, 9 USPQ2d 1461 (Fed. Cir. 1989) (commercial suitability not relevant to operability); *Ex parte Cole*, 223 USPQ 94, 95 (PTO Bd. App. 1983 ("We know of no statutory or case law requiring each and every compound within a claim to be equally useful for each and every contemplated application.")

Moreover, while we would agree that *these* articles do not teach transgenic fish that express fluorescence under sunlight (claim 1), they certainly *do* teach fish that express

fluorescence under UV light, and have a fluorescence that would meet the limitations of claim 43.

Turning to the "evidence" of general non-enablement relied upon by the Examiner, the only scientific article that is commented upon is that of Gong *et al*, which we will address below. The remaining articles, Moss *et al.*, Higashijima *et al.*, Kuo *et al.*, Kim *et al.* and Hackett, if anything, actually support enablement. For example, Moss *et al.* (Exhibit 2) would appear to *support* the enablement of claim 43 in that it discloses the use of a rat myosin light chain (MLC) promoter that successfully drives the detectable transient expression of green fluorescent protein in transgenic zebrafish embryos. The authors conclude that "[n]otably, the rat MLC transcription control regions present in our construct were capable of directing muscle-specific expression of the *gfp* in the zebrafish." Page 97, col. 2. Similarly, Higashijima *et al.* (Exhibit 3) discloses the use of α-actin and β-actin muscle promoters to drive the detectable expression of fluorescence genes in zebrafish; Kuo *et al.* (Exhibit 4) relates to the use of a neuron-specific promoter from a mammalian nectin gene, which is shown to faithfully direct expression of heterologous reporter genes in zebrafish neuronal tissue; Likewise, Kim *et al.* (Exhibit 5) demonstrates neuron-specific expression of a chicken gicerin cDNA in transient transgenic zebrafish using a mouse neurfilament promoter (NFP). Lastly, the Hackett review article (Exhibit 6) simply reviews technology relating to transgenic zebrafish production, as it stood in 1993. Thus, if anything, the foregoing articles actually support enablement.

Turning to the Gong reference (Exhibit 7), this reference also stands for broad enablement of claim 43, and also dependent claim 1. Gong discloses many of the studies that underlie the examples of the present application, but extends these studies to additional

fluorescence genes. Gong goes on to note that green fluorescent protein expressing transgenic zebrafish have been produced using many different tissue specific promoters, and cites to 8 different references in the reference section for this proposition. See page 62, col. 2, at top. Each of these references appears to describe transgenic zebrafish that express fluorescence detectable, for example, using a fluorescent light.

While it is agreed that Gong stands for the proposition that truly exceptionally high expression can be achieved, in part, by using a strong muscle promoter, we would direct the Board's attention to the statements regarding this publication from paragraph 8 of Dr. Gong's declaration: "As can be seen from reading the excerpt [at page 62, col.2] referred to by the examiner, it merely stands for the proposition that 'one' consideration is the strength of the promoter, and that another consideration is the tissue specificity, with muscle promoters in general being preferred for this reason. However, nowhere does the article in any way state or imply that the MLC2 promoter is "vital" to producing our fluorescent transgenic fish. As explained above, we know that this particular promoter is not 'vital' in this regard."

Lastly, turning to dependent claim 1, Appellants find no *prima facie* basis for maintaining such a rejection. As conceded by the Examiner, we have set forth two working examples in the specification of transgenic fish that express a fluorescence gene at a high enough level to be seen in sunlight. While there are indeed examples of transgenic fish in the prior art that do not meet the limitations of the fish recited in claim 1 (Higashijima *et al.* being an example), there are also many examples of others who have, subsequent to our priority date, reported that sun-visible expression had been achieved (Kinoshita and Chou *et al.* being examples). Moreover, Dr. Gong has provided a detailed explanation as to how the specification enables, without undue

experimentation, the production of transgenic fish that could be used in the practice of claim 1. Moreover, the Examiner has not raised any evidence to rebut the testimony of Dr. Gong in this regard.

Applicants would note that in the paragraph bridging pages 9 and 10, the Action raises the issue of mating species of fish of one species to fish of another species. Presumably this recitation goes to enablement of claim 36-40, which include a step of mating of fish. It was not Appellants intention to claim a novel form of breeding. Thus, it is submitted that such claim will certainly be interpreted as covering only breeding of fish species that can conventionally be bred. Similarly, Appellants note a concern at the top of page 9 regarding multiple colors of fish. However, this rejection is simply not understood. Appellants will attempt to address this rejection in their Reply Brief if the Examiner would be so kind as to clarify. In any event, Appellants would note that the Gong publication describes fish obtained using combinations of color genes, using the methodology of the present invention.

Accordingly, in view of the foregoing, the Board is requested to overturn the Examiner's enablement rejection of the claims.

B. Whether claims 1-3, 9-15, 20-21, 24, 30-32, 35-44 and 45 are properly rejected under 35 U.S.C. §112, first paragraph (lack of written description)

Next, all of the pending claims are rejected under 35 U.S.C. §112, first paragraph, on the basis of written description. Appellants have reviewed and considered this rejection but fail to understand its basis, or how it is different from the enablement rejection addressed above, in that the Examiner's comments appear to be consistent with the enablement issue, not written

description ("This analysis is based on whether specification teaches essential or critical elements or which are not adequately described in the specification and which are not conventional in the art as of the applicants' effective filing date for genus of muscle specific promoter derived from any species that would be strong enough to display fluorescence under normal sunlight." Action at page 15).

To the extent that the Examiner is taking the position that the specification fails to disclose the genus of muscle specific promoters *per se*, we would note that the specification teaches the concept of using muscle specific promoters generically (see, *e.g.*, paragraphs [0009] – [0011]) and sets forth two specific, working examples of particular muscle specific promoters, the MCK and MLC promoters (see paragraphs [0019], [0021], [0023], [00025]-[0026], [0033]-[0034] and [0079]-[0090]). Moreover, the fact that numerous muscle specific promoters were well known in the art at the time of filing has not been questioned by the Examiner. See Action at page 15. Rather, the Examiner's position appears to be that the specification fails to identify all such muscle promoters that would particularly useful for expressing at a sufficient level to produce sunlight-visible fluorescence. *Id.*

In response, Applicants would first again note that the principal claim, claim 43, does not require that the transgenic fish produce sunlight-visible fluorescence. Thus, there does not appear to be any issue with respect to claim 43.

Dependent claim 1 does specify sunlight-visible expression. However, as explained by Dr. Gong in his declaration, there is no requirement *per se* for any *special* muscle specific promoter in order to practice the subject matter of claim 1. Dr. Gong testifies that it would be expected that *any* muscle specific promoter can be successfully employed for this purpose. See

paragraph 6, particularly in light of paragraphs 4-5 and 7. Thus, since the specification generically refers to muscle promoters *per se*, and sets forth two specific, working examples of such muscle promoters, and in view of Dr. Gong's testimony that he contemplates that any muscle promoter can be employed in the practice of the invention.

In response, it is first noted that there is no legal basis under the written description requirement that an applicant set forth in the claims the specific structures being claimed where, as here, the *class* of compounds being claimed are known to the prior art. Instructive in this regard is the Federal Circuit's recent decision in *Capon v. Eshhar v. Dudas*, 418 F.3d 1349, 76 USPQ2d 1078 (Fed. Cir. 2005). As the *Capon* court points out, there is no requirement under written description that a specification contain a detailed description of elements where those elements are well known to those in the field:

> The Board stated that "controlling precedent" required inclusion in the specification of the complete nucleotide sequence of "at least one" chimeric gene. Bd. op. at 4. The Board also objected that the claims were broader than the specific examples. Eshhar and Capon each responds by pointing to the scientific completeness and depth of their descriptive texts, as well as to their illustrative examples. The Board did not relate any of the claims, broad or narrow, to the examples, but invalidated all of the claims without analysis of their scope and the relation of claim scope to the details of the specifications.
>
> Eshhar and Capon both argue that they have set forth an invention whose scope is fully and fairly described, for the nucleotide sequences of the DNA in chimeric combination is readily understood to contain the nucleotide sequences of the DNA components. Eshhar points to the general and specific description in his specification of known immune-related DNA segments, including the examples of their linking. Capon points similarly to his description of selecting DNA segments that are known to express immune-related proteins, and stresses the existing knowledge of these segments and their nucleotide sequences, as well as the known procedures for selecting and combining DNA segments, as cited in the specification.
>
> Both parties argue that the Board misconstrued precedent, and that precedent does not establish a *per se* rule requiring nucleotide-by-nucleotide re-analysis when the structure of the component DNA segments is already known, or

readily determined by known procedures. The "written description" requirement implements the principle that a patent must describe the technology that is sought to be patented; the requirement serves both to satisfy the inventor's obligation to disclose the technologic knowledge upon which the patent is based, and to demonstrate that the patentee was in possession of the invention that is claimed. See *Enzo Biochem*, 296 F.3d at 1330 (the written description requirement "is the *quid pro quo* of the patent system; the public must receive meaningful disclosure in exchange for being excluded from practicing the invention for a limited period of time"); *Reiffin v. Microsoft Corp.*, 214 F.3d 1342, 1345-46 (Fed. Cir. 2000) (the purpose of the written description requirement "is to ensure that the scope of the right to exclude . . . does not overreach the scope of the inventor's contribution to the field of art as described in the patent specification"); *In re Barker*, 559 F.2d 588, 592 n.4 (CCPA 1977) (the goal of the written description requirement is "to clearly convey the information that an applicant has invented the subject matter which is claimed"). The written description requirement thus satisfies the policy premises of the law, whereby the inventor's technical/scientific advance is added to the body of knowledge, as consideration for the grant of patent exclusivity.

The descriptive text needed to meet these requirements varies with the nature and scope of the invention at issue, and with the scientific and technologic knowledge already in existence. The law must be applied to each invention that enters the patent process, for each patented advance is novel in relation to the state of the science. Since the law is applied to each invention in view of the state of relevant knowledge, its application will vary with differences in the state of knowledge in the field and differences in the predictability of the science.

For the chimeric genes of the Capon and Eshhar inventions, the law must take cognizance of the scientific facts. The Board erred in refusing to consider the state of the scientific knowledge, as explained by both parties, and in declining to consider the separate scope of each of the claims. None of the cases to which the Board attributes the requirement of total DNA re-analysis, *i.e.*, *Regents v. Lilly*, *Fiers v. Revel*, *Amgen*, or *Enzo Biochem*, require a re-description of what was already known. In *Lilly*, 119 F.3d at 1567, the cDNA for human insulin had never been characterized. Similarly in *Fiers*, 984 F.2d at 1171, much of the DNA sought to be claimed was of unknown structure, whereby this court viewed the breadth of the claims as embracing a "wish" or research "plan." In *Amgen*, 927 F.2d at 1206, the court explained that a novel gene was not adequately characterized by its biological function alone because such a description would represent a mere "wish to know the identity" of the novel material. In *Enzo Biochem*, 296 F.3d at 1326, this court reaffirmed that deposit of a physical sample may replace words when description is beyond present scientific capability. In *Amgen Inc. v. Hoechst Marion Roussel, Inc.*, 314 F.3d 1313, 1332 (Fed. Cir. 2003) the court explained further that the written description requirement may be satisfied "if in the knowledge of the art the disclosed function is sufficiently correlated to a particular, known structure." These evolving principles were applied in *Noelle v. Lederman*, 355 F.3d 1343, 1349 (Fed. Cir. 2004), where the court affirmed that

> the human antibody there at issue was not adequately described by the structure and function of the mouse antigen; and in *University of Rochester v. G.D. Searle & Co.*, 358 F.3d 916, 925-26 (Fed. Cir. 2004), where the court affirmed that the description of the COX-2 enzyme did not serve to describe unknown compounds capable of selectively inhibiting the enzyme.
>
> The "written description" requirement must be applied in the context of the particular invention and the state of the knowledge. The Board's rule that the nucleotide sequences of the chimeric genes must be fully presented, although the nucleotide sequences of the component DNA are known, is an inappropriate generalization. When the prior art includes the nucleotide information, precedent does not set a *per se* rule that the information must be determined afresh. Both parties state that a person experienced in the field of this invention would know that these known DNA segments would retain their DNA sequences when linked by known methods. Both parties explain that their invention is not in discovering which DNA segments are related to the immune response, for that is in the prior art, but in the novel combination of the DNA segments to achieve a novel result.
>
> The "written description" requirement states that the patentee must describe the invention; it does not state that every invention must be described in the same way. As each field evolves, the balance also evolves between what is known and what is added by each inventive contribution. Both Eshhar and Capon explain that this invention does not concern the discovery of gene function or structure, as in Lilly. The chimeric genes here at issue are prepared from known DNA sequences of known function. The Board's requirement that these sequences must be analyzed and reported in the specification does not add descriptive substance. The Board erred in holding that the specifications do not meet the written description requirement because they do not reiterate the structure or formula or chemical name for the nucleotide sequences of the claimed chimeric genes.

The Capon case has very recently been followed by the Federal Circuit, in *Falkner v. Inglis*, 79 USPQ2d 1001 (Fed. Cir. 2006). In a section of the opinion entitled "Recitation of Known Structure Is Not Required" the *Falkner* court, following the *Capon* decision, stated:

> Indeed, a requirement that patentees recite known DNA structures, if one existed, would serve no goal of the written description requirement. It would neither enforce the quid pro quo between the patentee and the public by forcing the disclosure of new information, nor would it be necessary to demonstrate to a person of ordinary skill in the art that the patentee was in possession of the claimed invention. ... Accordingly, we hold that where, as in this case, accessible literature sources clearly provided, as of the relevant date, genes and their nucleotide sequences (here "essential genes"), satisfaction of the written

> description requirement does not require either the recitation or incorporation by reference (where permitted) of such genes and sequences.

Id. at 1008.

The fact that tissue specific promoters were exceedingly well known as of the filing date is evidenced by evidence set forth above in the enablement section of this response.

Thus, it is submitted that there is clearly no *prima facie* basis for subject written description rejection, and that Applicants have provided evidence in support of written description. For these reasons, the Board is requested to withdraw the rejection.

C. Whether claims 1-3, 9-15, 20-21, 24, 30-32, 35-44 and 45 are properly rejected on the basis of obviousness-type double patenting of US 7,135,613.

Lastly, the Action rejects all of the pending claims (noting that the claim numbers of which are listed incorrectly) on the basis of obviousness-type double patenting ("ODP") over US 7,135,613. The basis of the rejection is unclear from the Action. In the previous Action, mailed June 10, 2008, it is simply stated that "both sets of claims encompass a transgenic fish comprising a chimeric gene comprising a muscle specific promoter that drives the expression of a structural gene in said fish ..." Action dated 6/10/08 at page 21. Appellants note that the claims of the '613 patent are directed to transgenic fish *per se*.

Appellants assert that the Action fails to set forth a *prima facie* ODP rejection, that the present claims are anticipated or obvious over the specified claims of the '613, or *vice versa*. See MPEP §804 B.1. As noted in MPEP §804 B.1., a double patenting rejection of the obviousness-type, if not based on an anticipation rationale, is "analogous to [a failure to meet] the nonobviousness requirement of 35 U.S.C. 103" except that the patent principally underlying

the double patenting rejection is not considered prior art. *In re Braithwaite*, 379 F.2d 594, 154 USPQ 29 (CCPA 1967). Therefore, the analysis employed in an obviousness-type double patenting rejection parallels the guidelines for analysis of a 35 U.S.C. 103 obviousness determination. *In re Braat*, 937 F.2d 589, 19 USPQ2d 1289 (Fed. Cir. 1991); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985).

Since the analysis employed in an obviousness-type double patenting determination parallels the guidelines for a 35 U.S.C. 103(a) rejection, the factual inquiries set forth in *Graham v. John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966), that are applied for establishing a background for determining obviousness under 35 U.S.C. 103 are employed when making an obvious-type double patenting analysis. MPEP §804 B.1. clearly states that any obviousness-type double patenting rejection should make clear:

> (A) The differences between the inventions defined by the conflicting claims - a claim in the patent compared to a claim in the application; and
>
> (B) The reasons why a person of ordinary skill in the art would conclude that the invention defined in the claim at issue is anticipated by, or would have been an obvious variation of, the invention defined in a claim in the patent.

Moreover, when considering whether the invention defined in a claim of an application would have been an obvious variation of the invention defined in the claim of a patent, the disclosure of the patent may not be used as prior art. *General Foods Corp. v. Studiengesellschaft Kohle mbH*, 972 F.2d 1272, 1279, 23 USPQ2d 1839, 1846 (Fed. Cir. 1992).

No such analysis has even been attempted here.

As an aside, Appellants would observe that during the prosecution of USSN 09/913,898, Applicants attempted to introduce claims consistent with the claims pending in the present application. See Applicant's Amendment dated May 9, 2003. (Exhibit 8). In response to this attempted amendment, the Examiner refused entry of the amendment: "The amendment filed on May 12, 2003 ... presenting only claims drawn to a new invention is **non-responsive** (MPEP §821.03) (underline added)," taking the position that such claims were found not to be drawn to the invention elected in that case, which later became the '613 patent. See Restriction Requirement mailed 12/18/03 and, again, the Restriction Requirement dated 7/30/03. (Exhibit 9) Thus, the PTO has already decided that the present claims are patentably distinct.

In view of the foregoing, Appellants respectfully request that the Board reverse the rejections of all claims.

Respectfully submitted,

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VIII. Claims Appendix

1. The method of claim 43, further defined as comprising the steps of:

(a) obtaining a transgenic fish line comprising one or more chimeric genes that are positioned under the control of a promoter that drives the expression of a fluorescent protein in muscles of said fish, said promoter being a muscle specific promoter, such that said transgenic fish expresses fluorescent protein encoded by the gene in skeletal muscle at a level sufficient such that said transgenic fish fluoresces upon exposure to sunlight, wherein said transgenic fish are the offspring of an embryo line visually exhibiting expression of the fluorescent protein in essentially all muscle fibers in their trunk and further wherein transgenic founders of said line fluoresce upon exposure to sunlight; and

(b) distributing fish of said line to the ornamental fish market.

2. The method of claim 1 or claim 43, further comprising displaying said transgenic fish under a blue or ultraviolet light.

3. The method of claim 2, wherein the transgenic fish are displayed under an ultraviolet light that emits light at a wavelength selected to be optimal for the fluorescent protein or proteins.

4. – 8. (Cancelled)

9. The method of claim 1 or claim 43, wherein the transgenic fish express a BFP.

10. The method of claim 9, wherein the transgenic fish express an EBFP.

11. The method of claim 1 or claim 43, wherein the transgenic fish express a YFP.

12. The method of claim 11, wherein the transgenic fish express an EYFP.

13. The method of claim 1 or claim 43, wherein the transgenic fish express a CFP

14. The method of claim 13, wherein the transgenic fish express an ECFP.

15. The method of claim 1 or claim 43,
wherein the transgenic fish expresses more than one color of fluorescent protein encoded by the gene or genes.

16. – 19. (Cancelled)

20. The method of claim 1 or claim 43, wherein the promoter is a zebrafish muscle creatine kinase gene promoter.

21. The method of claim 1 or claim 43, wherein the promoter is a zebrafish myosin light chain 2 gene promoter.

22. – 23. (Cancelled)

24. The method of claim 1 or claim 43, wherein one or more of said chimeric genes further comprises a ubiquitously expressing promoter.

25. – 29. (Cancelled)

30. The method of claim 15, wherein the more than one fluorescent protein is expressed in the same tissue, to effect a new fluorescent color.

31. The method of claim 30, where the transgenic fish expresses a GFP and a BFP.

32. The method of claim 15, wherein the more than one fluorescent proteins are separately expressed in different tissues.

33. – 34. (Cancelled)

35. The method of claim 32, wherein the transgenic fish expresses a YFP under the control of a muscle specific promoter.

36. The method of claim 1 or claim 43, wherein the transgenic fish is a stable transgenic fish line obtained by a method comprising the steps of:

(a) obtaining a transgenic fish comprising one or more fluorescence genes positioned under the control of a promoter, wherein the transgenic fish expresses one or more fluorescent proteins encoded by the one or more fluorescence genes; and

(b) breeding the transgenic fish with a second fish to obtain offspring; and

(c) selecting from said offspring a stable transgenic line that expresses one or more fluorescent proteins.

37. The method of claim 36, wherein the second fish is a wild type fish.

38. The method of claim 36, wherein the second fish is a second transgenic fish.

39. The method of claim 1 or claim 43, wherein the transgenic fish is a transgenic zebrafish, medaka, goldfish or carp.

40. The method of claim 36, wherein the second fish is a zebrafish, medaka, goldfish or carp.

41. The method of claim 1, 36 or 43, wherein the transgenic fish is a transgenic koi, loach, tilapia, glassfish, catfish, angel fish, discus, eel, tetra, goby, gourami, guppy, Xiphophorus, hatchet fish, Molly fish, or pangasius.

42. The method of claim 39, wherein the transgenic fish is a transgenic zebrafish.

43. A method of providing transgenic fish to the ornamental fish market, comprising the steps of:

(a) obtaining a transgenic fish line comprising one or more chimeric genes that are positioned under the control of a promoter that drives the expression of a fluorescent protein in muscles of said fish, said promoter being a muscle specific promoter, such that said transgenic fish expresses fluorescent protein encoded by the gene in skeletal muscle at a level sufficient such that said transgenic fish fluoresces upon exposure to one or more light; and

(b) distributing fish of said line to the ornamental fish market.

44. The method of claim 1 or 43, wherein the transgenic fish express a GFP.

45. The method of claim 44, wherein the transgenic fish express an EGFP.

IX. EVIDENCE APPENDIX

EXHIBIT 1. The Declaration of Zhiyuan Gong, Ph.D. ("Gong Declaration") and its referenced exhibit ("Declaration Exhibit 1"), made of record by the Office Action Response dated October 7, 2008.

EXHIBIT 2. Moss *et al.*, made of record by the Information Disclosure Statement (Form 1449) dated November 28, 2003.

EXHIBIT 3. Higashijima *et al.*, made of record by the Information Disclosure Statement (Form 1449) dated November 28, 2003.

EXHIBIT 4. Kuo *et al.*, made of record by the Information Disclosure Statement (Form 1449) dated November 28, 2003.

EXHIBIT 5. Kim *et al.*, made of record by the Information Disclosure Statement (Form 1449) dated November 28, 2003.

EXHIBIT 6. Hackett *et al.*, made of record by the Information Disclosure Statement (Form 1449) dated November 28, 2003.

EXHIBIT 7. Gong *et al.*, made of record in the Office Action dated December 10, 2008.

EXHIBIT 8. Applicant's Amendment, presented in the Office Action Response dated May 9, 2003.

EXHIBIT 9. Office Action and communication, mailed on December 18, 2003 (Office Action including a restriction/election requirement) and July 30, 2003 (communication further regarding the election of claims).

Exhibit 1

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Gong et al.	Group Art Unit: 1632
Serial No.: 10/605,708	Examiner: Singh, Anoop Kumar
Filed: October 21, 2003	Atty. Dkt. No.: GLOF:007USC1
For: CHIMERIC GENE CONSTRUCTS FOR GENERATION OF FLUORESCENT TRANSGENIC ORNAMENTAL FISH	Confirmation No: 2707

DECLARATION OF ZHIYUAN GONG, PH.D.

I, Zhiyuan Gong, hereby declare as follows:

1. I am a co-inventor of the subject disclosed and claimed in the referenced application and I am familiar with the contents of said application. I am currently a professor of Biological Sciences, National University of Singapore, Singapore. I have extensive training and experience in the field of transgenic fish, as evidenced by my attached *curriculum vitae*, a copy of which is attached as Exhibit 1.

2. I understand that the PTO examiner in charge of examining the referenced patent application has rejected the pending claims, taking the position that the specification does not reasonably provide enablement for using any muscle specific promoter other than exemplified muscle specific promoters to obtain stable transgenic fish line suitable for ornamental fish marker showing fluorescence upon exposure to sunlight. I am submitting this declaration disagreeing on the enablement rejection for the following reasons.

3. The expression of identical transgenes that insert into different regions of a genome or a transgenic organism is variable, which is described as the "position effect." In this case the difference in expression is often due to local regulatory sequences that regulate neighboring genes or influences of local environment. Due to the position effect, a transgene, even under a strong promoter, will be expressed poorly, if at all, if it falls within a heterochromatin area or is silenced by a local regulatory sequence. To the contrary, a transgene, even under a weak promoter, could be expressed at a high level if its expression is enhanced by a local regulatory sequence, depending on the positional context.

4. It has been my experiences that a wide variation of expression is common for expressing transgenes in fishes, even where known strong promoters are employed to drive expression. Given the enormous number of potential sites of integration in a fish genome, expression of a transgene may be frequently affected by the position effect due to random integration of a transgene. Since not all transgenic constructs will behave in the manner that may be desired, a screening process for those fish with the desired level of transgene expression will be useful.

5. I have reviewed and am familiar with the specification of the referenced patent application, and would note that in Example III the specification teaches that one can screen the transgenic fish embryos to select those embryos exhibiting the desired expression characteristics. Particularly, preferred are those embryos exhibiting high expression such that the fluorescence is visible in the sunlight. I would also direct attention to Figures 8 through 12, particularly Figure 12, and their associated figure legends. The reason for using such an embryo selection technique (or any other suitable selection technique) is to identify a founder embryo that is likely

to give rise to a highly fluorescent line of fish that, if desired, express brightly enough to exhibit visible fluorescent coloration, visible even in sunlight.

6. Based on my knowledge and experience in the production of fluorescent, transgenic fish, it is my opinion that virtually any muscle-specific promoter can be employed to produce very highly fluorescent founder embryos and lines. While it may well be necessary in some instances to use one of the above or other screening procedure that permits one to select those embryos that have appropriate position effects, this should require only reasonably routine repetitive steps. Of course, when a weaker promoter is employed it may be necessary to inject and screen larger numbers of embryos, which may be more than one thousand, to identify a "high expresser" but again such screening is straightforward and does not involve any additional inventiveness to accomplish. Considering the muscle occupies a large part of the fish body and thus has the capacity to synthesize enough proteins for visible fluorescence, screening for visible fluorescence using any muscle-specific promoter provides specific guidance and predictable results for obtaining stable transgenic fish suitable for ornamental fish market.

7. I would further direct the examiner's attention to the attached article of Kinoshita entitled "Transgenic medaka with brillaint fluorescence in skeletal muscle under normal light" (*Fisheries Science, 70*:645-649, 2004). As the title implies, this article describes the preparation of transgenic, fluorescent medaka having a brilliant fluorescence in skeletal muscle under normal light. In these studies, the author employed the skeletal muscle actin promoter. Further, on page 648, col. 1, the author also mentions the article of Chou *et al.* (*Transgenic Res., 10*: 303-315, 2001), which is said to teach transgenic medaka strains with the GFP gene under the control of the β-actin gene regulatory region, which could be observed under daylight.

8. I further understand that in connection with the above-mentioned rejection, the examiner relies on a statement from page 62, col. 2, of my 2003 BBRC publication, with respect to which the examiner states that:

> It is clear from the teaching of Gong et al that strong expression of a fluorescent gene under the control of MLC2 promoter in muscle tissue that constitutes majority of the fish body tissue is vital for successfully generating transgenic fish for distribution in ornamental fish market.

This is not a true statement. As can be seen from reading the excerpt referred to by the examiner, it merely stands for the proposition that "one" consideration is the strength of the promoter, and that another consideration is the tissue specificity, with muscle promoters in general being preferred for this reason. However, nowhere does the article in any way state or imply that the MLC2 promoter is "vital" to producing our fluorescent transgenic fish. As explained above, we know that this particular promoter is not "vital" in this regard.

9. I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

23/9/08

Date

Zhiyuan Gong

Declaration

Exhibit 1

CURRICULUM VITAE

Name: **Zhiyuan Gong** Date: Dec. 2006
Date of Birth: April 6, 1959
Sex: Male

EDUCATION AND PROFESSIONAL EXPERIENCE:

July 2007--present. Professor, Department of Biological Sciences, National University of Singapore, Singapore

July 2006—June 2007, Associate Professor, Department of Biological Sciences, National University of Singapore, Singapore

July 2005-June 2006, Sabbatical leave at Department of Molecular, Cellular and Developmental Biology, University of California at Los Angeles, USA,

July 1997--June 2000. Senior Lecturer, Department of Biological Sciences, National University of Singapore, Singapore

June. 1995-- June 1997. Lecturer, Department of Biological Sciences, National University of Singapore, Singapore

Nov. 1988--June 1995. Research Fellow. Research Institute, Hospital for Sick Children, Toronto, and Departments of Biochemistry and Clinical Biochemistry, Banting Institute, University of Toronto.

Oct. 1987 -- Oct. 1988. Postdoctoral fellow, Department of Biology, McGill University, Montreal.

1984 (summer). Trainee in Embryology Course, Marine Biological Laboratory, Woods Hole, MA, U.S.A.

Sept. 1983 -- Oct. 1987. Department of Biology, McGill University, Montreal. **Ph.D. (1987), Dean's Honour List**.

Feb. 1982 -- Aug. 1983. Institute of Genetics and Department of Biology, Fudan University, Shanghai, China.

Feb. 1978 -- Jan. 1982. Department of Marine Biology, Ocean University of Qingdao (Shandong Oceanology College), Qingdao, China. **B.Sc. (1982)**

B. RESEARCH

I. MAJOR RESEARCH PROGRAMMES

Before Joining NUS

1. PH.D Project: Tubulin gene regulation in sea urchin embryos (1983-1987). I have demonstrated that tubulin genes are autogenously regulated by the concentration of free tubulin subunits in sea urchin embryos and the autogenous regulation plays an important role in developmental regulation of tubulin gene expression. This is the first demonstration of tubulin gene autoregulation in an in vivo system. The mechanism of the autoregulation is posttranscriptional at the level of RNA stability. In addition, I found that tubulin gene transcription could be stimulated by deciliation (removal of cilia). Five major papers have been published from this subject.

2. Postdotoral Research Projects (University of Toronto, 1988-1995)
In my postdoctoral training at University of Toronto, I was mainly involved in fish antifreeze protein gene project. While it was commonly believed that fish AFPs are produced exclusively in the liver and secreted into the blood for extracellular function, I found that the non-liver tissues also express AFP mRNAs in several species of fish and that non-liver AFP genes are regulated differently in response to the seasonal change and hypophysectomy. Furthermore, I have cloned several non-liver AFP genes and found that these non-liver genes encode AFPs without the signal peptide and prosequence which are commonly present in the liver AFPs. Therefore, the non-liver AFPs are likely to function intracellularly. This study opens a new area for antifreeze gene research in gene regulation and evolution. In addition, I was also involved in transgenic fish studies and cloning of pituitary hormone genes from several fish species.

Research Programs Developed in NUS

Since I joined NUS, I mainly used the zebrafish model for research. The zebrafish model was originally used for developmental analyses. To align with national R and D agenda, I also developed the zebrafish model for applications in biotechnology, environmental and medical sciences.

1. Zebrafish in Developmental Biology
Molecular dissection of neurogenic pathway in zebrafish embryos (NUS, since 1995). In the past few years, my laboratory has isolated many zebrafish cDNA clones involving in neural development. These cDNA clones belong to several gene families including *gli* zinc finger, *iroquois* homeobox, bHLH, and LIM homeobox families. By collaboration with Dr. V. Korzh, we have been actively characterizing their expression and function in developing zebrafish embryos and several mutants. Our current working hypothesis on the neurogenic pathway is: *sonic hedgehog* --> *gli/zic* zinc finger genes -->*iroquois*

homeobox genes --> bHLH genes --> LIM homeobox genes. While the work is still in progress, 12 papers have already published in major developmental biology journals such as *Development, Mechanism of Development* and *Developmental Dynamics.*

Zebrafish EST project (NUS, 1995-1998). In order to rapidly build up the genetic resources to facilitate molecular analyses in the increasingly important zebrafish model, our group was the first one to launch a zebrafish EST project for rapid isolation and identification zebrafish cDNA clones. Over the years from 1995 to 1998, we had generated over 3,000 zebrafish EST clones from several cDNA libraries including embryonic, whole adult, brain, eye, ovary and testis cDNA libraries. Our work was particularly important at the early stage of zebrafish EST project and helped to map the zebrafish genome before the initiation of the NIH funded zebrafish EST project in the large sequencing center in Washington University in 1998. During this period, we had received nearly 200 requests for zebrafish cDNA clones and libraries from all over the world. Recently, we have contributed two zebrafish gonad cDNA libraries (now named Gong Ovary and Gong Testis by the zebrafish community) to the international zebrafish EST project and over 25,000 EST clones were generated from these two libraries. 1,090 of these sequences have been used in the unigene set (16,000 genes) for designing of zebrafish microarray oligonucleotides by Compugen.

GFP transgenic zebrafish (NUS, since 1998). With the aid of our zebrafish EST project and an improved linker-mediated PCR method we developed, we were able to rapidly isolate gene promoters based on the EST clones. So far, we have isolated many tissue-specific and inducible promoters. These promoters have been linked with the GFP gene and introduced into zebrafish. We have generated dozens of stable transgenic lines for skin specificity, muscle specificity, liver specificity, exocrine pancreas specificity, oocyte specificity and neuron specificity. These transgenic lines are important assets for further analysis of promoters and gene expression programs, tracing cell lineage and migration, use for cell and nuclear transplantation etc. Some of the transgenic lines have two colors (GFP and RFP) for easy tracing development of multiple organs/tissues.

Evolutionary comparison of development of zebrafish swim bladder and tetrapod lung
As a member of SMA (Singapore-Massachusetts Institute of Technology), I proposed to work on development of zebrafish endodermal organs. The liver development is supported by the BMRC grant and the intestine organ is currently conducted by SMA students. Now I propose to work on swim bladder development. The swim bladder is a fish homolog of tetrapod lung. Comparison of development of swim bladder and lung will pose exciting questions not only in development but also in evolution. In embryos, both lung and swim bladder arise from an outgrowth of the gastrointestinal tract. The main function of the lung is to breathe air while the swim bladder can be inflated and deflated to change buoyancy in water. Furthermore, some lung-specific molecules such as surfactant proteins are also found in the swim bladder. Thus, the tetrapod lung and fish swim bladder are likely to share a common evolutionary origin. This proposal aims at understanding of the development and evolution of the two organs. In particular, we propose to compare expression pattern of important genes in lung and swim bladder development, and thus to identify the critical genes responsible for the distinction of developmental pathways for the two organs. The detailed characterization of fish swim

bladder, less vulnerable to infection, should also provide new insight into human lung development and lung infection.

Zebrafish in Biotechnology, Environmental and Medical Sciences

Fluorescent transgenic ornamental fish and transgenic fish bioreactor (NUS, since 1998). By strong expression of several fluorescent protein genes, including green, yellow and red fluorescent protein genes, in the skeletal muscle of zebrafish, we demonstrated that the fluorescent colors can be viewed by unaided eyes and thus it is feasible to use the transgenic technology to generate novel varieties of ornamental fish. Furthermore, by crossing with two transgenic lines of different color, we can generate new and intermediate colors, thus increasing the capacity of generating more transgenic colors. We also demonstrated that these transgenic fish can express recombinant proteins up to 17% of total muscle proteins, thus the muscle expression in transgenic fish may be used as a new transgenic bioreactor system. The technology for generation of fluorescent ornamental fish has been patented and licensed to an US company with a trademark **"GloFish".** The GloFish is being marketed in USA as the first genetically modified pet and received intensive attention worldwide, with a wide coverage in virtually all major global media outlets, including *CNN, BBC, CNBC, Fox, NHK, Newsweek, Nature, New Scientists, Washington Post New York Times, The Wall Street Journal, etc.*, with >500,000,000 media impressions (e.g. *Nature* **426**:372 and 596 [2003]; *Nature Biotechnology* **22**:1 [Editorial]; **22**:11 [2004]). It was also a debated topic in USA presidential election 2004 (*Nature.* 2004 Sep 16;431(7006):238-243). The significance of GloFish™ reaches far beyond the ornamental fish industry, as they have become a biotechnology showcase. In addition to their introduction into public aquaria for science education, GloFish™ have also been used as teaching materials in universities and high schools. As a biotechnology pioneer, GloFish™ will help the general public to establish faith in biotechnology products and will surely have a place in the history of biotechnology. Now the term GloFish has been included in Wikipedia, a web encyclopaedia, and *Singapore Encyclopaedia.* Because of our pioneer work, a new field of research is emerging by using our transgenic fish to evaluate the ecological effects of transgenic fish. Currently, we are also developing transgenic fish to express vaccines and to prove the concept of sushi-type edible vaccine.

Development of transgenic biomonitoring fish for environmental protection (NUS, since 1999). We have also generated transgenic biomonitoring fish using some inducible promoters to drive transgenic expression of fluorescent protein gene. Currently we are aiming at monitoring of endocrine disrupters and heavy metals. Both estrogen-responsive and heavy metal-inducible promoters have been isolated and engineered with the fluorescent protein genes. Stable transgenic lines have been developed for both zebrafish and medaka. Preliminary characterization of these transgenic fish indicated inducibility of GFP expression by many of the environmental relevant compounds. This work has made a finalist for Asian Innovation Award by *Far East Economic Review* (2002).

NEWater – Health effect studies using medaka fish as a model (Singapore national project, 1999-2003). Due to the shortage of water supply and the threat of cut-off from a neighbour country, Singapore government has launched a national water reclamation project using the membrane and reverse osmosis technology. The project was initiated in

1999 and I was appointed as the principle investigator by a national committee to conduct the fish study to test the health effect in reclaimed water (NEWater). In this project, we are using the medaka fish to conduct tumor and estrogenic tests. Because this is an unprecedented study, we have to develop all standard research protocols and all studies were carried out under stringent QA/QC monitoring. Two fish tests have been successfully conducted and a confidential report has been submitted through Ministry of Environment to the Prime Minister's Office for policy making.

Zebrafish liver program (NUS-BMRC project, since 2002). As the program coordinator and principle investigator, together with eight other co-PIs from DBS, Chemistry, Biochemistry, Genome Institute of Singapore and Institute of Molecular and Cell Biology, we have successfully won $5.3 BMRC (Biomedical Research Council of Singapore) co-operative research grant from the first round of national competition. In this project, we are focusing on molecular mechanisms of liver carcinogenesis and development using the zebrafish as a model. Other than coordinating the research activities among different groups across a few institutions/universities, I am leading my research to focus on development of zebrafish genomic tools, including generation of EST/full length cDNA sequences; production of zebrafish DNA chips using 60-mer oligonucleotides representing unigenes or singltons; investigation of zebrafish gene expression in response to environmental pollution and development of zebrafish DNA chip for environmental monitoring; generation of liver tumors by carcinogen treatment as well as by transgenic expression of oncogenes; and generation of liver and other tissue-specific GFP transgenic lines. By these studies, we wish to identify novel zebrafish genes involved in liver carcinogenesis and development. So far, we have published over 25 research papers under this program and, in particular, two of them appeared in top journals, *PLOS Genetics* and *Nature Biotechnology*. Our landmark works validated the zebrafish model for human disease studies by comparative analysis of transcriptome profiles in human and zebrafish liver cancers.

II. RESEARCH CONTRIBUTIONS:

A. Peer Reviewed Primary Research Papers published in international journals:

1. Floyd, E.E., **Z. Gong**, B.P. Brandhorst and W. H. Klein (1986) Calmodulin gene expression during sea urchin development: Persistence of a prevalent maternal protein. *Dev. Biol.* **103**:501-511.
2. **Gong, Z.** and B.P. Brandhorst (1987) Stimulation of tubulin gene transcription by deciliation of sea urchin embryos. *Mol. Cell. Biol.* 7:4238-4246.
3. **Gong, Z.** and B.P. Brandhorst (1988) Autogenous regulation of tubulin synthesis via RNA stability during sea urchin embryogenesis. *Development* **102**:31-43.
4. **Gong, Z.** and B.P. Brandhorst (1988) Stabilization of tubulin messenger RNA by inhibition of protein synthesis in sea urchin embryos. *Mol. Cell. Biol.* **8**:3518-3525.
5. **Gong, Z.** and B.P. Brandhorst (1988) Multiple levels of regulation of tubulin gene expression during sea urchin embryogenesis. *Dev. Biol.* **130**:144-153.
6. **Gong, Z.** and B.P. Brandhorst (1988) Microtubule formation from maternal tubulins during sea urchin embryogenesis: Measurement of Soluble and insoluble tubulin pools. *Mol. Reprod. Dev.* **1**:3-9.
7. **Gong, Z.**, P. Cserjesi, G.M. Wessel, and B.P. Brandhorst (1991) Structure and expression of the polyubiquitin gene in sea urchin embryos. *Mol. Reprod. Dev.* **28**:111-118.
8. **Gong, Z.**, C.L. Hew, and J.R. Vielkind (1991) Functional analysis and temporal expression of promoter regions from fish antifreeze protein genes in transgenic Japanese Medaka embryos. *Mol. Marine Biol. Biotech.* **1**: 64-72.
9. Lu, M., **Z. Gong**, W. Shen, and A.D. Ho (1991) The *Tcl*-3 proto-oncogene altered by chromosomal translocation in T-cell leukemia codes for a homeobox protein. *EMBO J.* **10**:2905-2910.
10. **Gong, Z.** (1992) Improved RNA staining in formaldehyde gels. *BioTechniques* **12**:74-76.

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11. Du, S.J., **Z. Gong**, G.L. Fletcher, M.A. Shears, M.J. King, D.R. Idler, and C.L. Hew (1992) Growth enhancement in transgenic Atlantic salmon by use of fish antifreeze/growth hormone chimeric gene constructs. *Bio/Technology* **10**:176-181.
12. Elsholtz, H.P., S. Majumdar-Sonnylal, F. Xiong, **Z. Gong** and C.L. Hew (1992) Phylogenetic specificity of prolactin gene expression with conservation of pit-1 function. *Mol. Endocrin.* **6**:515-522.
13. **Gong, Z.**, G.L. Fletcher and C. L. Hew (1992) Tissue distribution of fish antifreeze protein mRNAs. *Can. J. Zool.* **70**:810-814.
14. Du, S.J., **Z. Gong**, C.H. Tan, G.L. Fletcher and C.L. Hew (1992). The design and construction of "all fish" gene casette for aquaculture. *Mol. Marine Biol. Biotech.* **1**:290-300.
15. Iraqi, F., **Z. Gong**, L. Crim and C.L. Hew. (1993) Isolation and characterization of somatolactin genes from two cold water marine teleosts, lumpfish (*Cyclopterus lump*) and halibut (*Hippoglossus hippoglossus*). *Mol. Marine Biol. Biotech.* **2**: 96-103.

16. **Gong, Z.**, Z. Hu, Z. Q. Gong, R. Kitching, and C.L. Hew. (1994) Bulk isolation and identification of fish genes by cDNA clone tagging. *Mol. Marine Biol. Biotech.* **3**:243-251.
17. **Gong, Z.** and C.L. Hew. (1994) Zinc and DNA binding properties of a novel LIM domain homeobox protein Isl-2. *Biochemistry* **33**:15149-15158.
18. **Gong, Z.**, M.J. King, G.L. Fletcher, and C.L. Hew (1995) The antifreeze protein genes of the winter flounder, Pleuronectus americanus, are differentially regulated in liver and non-liver tissues. *Biochem. Biophys. Res. Commun.* **206**:387-392.
19. **Gong, Z.**, C.-c. Hui, and C.L. Hew (1995). Presence of *Isl-1* related LIM domain homeobox genes in teleost: their similar patterns of expression in brain and spinal cord. *J. Biol. Chem.* **270**: 3335-3345.
20. Wang, R., P. Zhang, **Z. Gong**, C.L. Hew (1995) The expression of antifreeze protein gene in transgenic goldfish (Carassius auratus) and its implication in cold adaptation. *Mol. Marine Biol. Biotech.* **4**: 20-26.
21. **Gong, Z.**, and C.L. Hew (1995) Several splicing variants of *isl-1* like genes in the chinook salmon (*Oncorhynchus tschawytscha*) encode truncated transcription factors containing a complete LIM domain. *Biochim. Biophy. Acta* **1260**:349-354.

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22. Tokumoto, M., **Z. Gong**, T. Tsubokawa, C.L. Hew, K. Uyemura, Y. Hotta, and H. Okamoto (1995) Molecular heterogeneity among primary motoneurons and within myotomes revealed by the differential mRNA expression of novel Islet homologs in embryonic zebrafish. *Dev. Biol.* **171**: 578-589.

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25. Liao, J. and **Z. Gong** (1997) Sequencing of 3' cDNA clones using anchored oligo dT primers. *BioTechniques* **23**:368-370.
26. **Gong, Z.**, T. Yan, J. Liao, S.E. Lee, J. He and C.L. Hew (1997) Rapid identification and isolation of zebrafish cDNA clones. *Gene* **201**:87-98.
27. He, J., Z. Yin, G. Xue, **Z. Gong**, T.J. Lam and Y.M. Sin (1997) Production of goldfish against *Ichthyophthrius multifilis* by immunization with a recombinant vaccine. *Aquaculture* **158**:1-10.
28. Liao, J., C.H. Chan and **Z. Gong** (1997) An alternative linker-mediated polymerase chain reaction method using a dideoxynucleotide to reduce amplification background. *Anal. Biochem.* **253**:137-139.

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29. Yan, T., and **Z. Gong** (1998) Assembly of a complete zebrafish mitochondrial 16S rRNA gene from overlapping expressed sequence tags. *DNA Sequence* **9**:145-148..
30. Lim, J.H., J. He, V. Korzh and **Z. Gong** (1998) A new splicing variant of a type III POU gene from zebrafish encodes a POU protein with a distinct C-terminal. *Biochim. Biophy. Acta* **1397**:253-256.
31. Postlethwait, J.H., Y.-L. Yan, M.A. Gates, S. Horne, A. Amores, A. Brownlie, A. Donovan, E.S. Egan, A. Force, **Z. Gong**, C. Goutel, A. Fritz, R. Kelsh, E. Knapik, E. Liao, B. Paw, D. Ransom, A. Singer, M. Thomson, T.S. Abduljabbar, P. Yelick, D. Beier, J.-S. Joly, D. Larhammar, F. Rosa, M. Westerfield, L.I. Zon, S. Johnson and W. Talbot (1998) Vertebrate genome evolution and the zebrafish map. *Nature Genetics* **18**:345-349.
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33. Miao, M., S.-L. Chan, C.L. Hew and **Z. Gong** (1998) The skin-type antifreeze protein gene intron of the winter flounder is a ubiquitous enhancer lacking a functional C/EBPα binding motif. *FEBS Letters* **426**: 121-125.

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34. Xu, Y., J. He, H.L. Tian, C.H. Chan, J. Liao, T. Yan, T.J. Lam and **Z. Gong** (1999) Fast skeletal muscle specific expression of a zebrafish myosin light chain 2 gene and characterization of its promoter by direct injection into skeletal muscle. *DNA Cell Biol.* **18**:85-95.
35. Liao, J., J. He, T. Yan, V. Korzh and **Z. Gong** (1999) A class of NeuroD-related basic helix-loop-helix transcription factors which are expressed in developing central nervous systems in zebrafish. *DNA Cell Biol.***18**:333-344.
36. Xu, Y. and **Z. Gong** (1999) Adaptation of inverse PCR to generate an internal deletion. *Biotechniques*. **26**:639-641
37. Ju, B., Y. Xu, J. He, J. Liao, T. Yan, C.L. Hew, T.J. Lam and **Z. Gong**. (1999) Faithful expression of green fluorescent protein (GFP) in transgenic zebrafish embryos under zebrafish gene promoters. *Dev Genetics*. **25**:158-167.
38. Garg, R.R., L. Bally-Cuif, S.E. Lee, **Z. Gong**, X. Ni, C.L. Hew and C. Peng (1999) Cloning of zebrafish activin type IIB receptor (ActRIIB) cDNA and mRNA expression of ActRIIB in embryos and adult tissues. *Mol. Cell. Endocrinol.* **153**:169-181.
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41. Wang, H. and **Z. Gong** (1999). Characterization of two zebrafish cDNA clones encoding egg membrane proteins ZP2 and ZP3. *Biochim. Biophy. Acta.* **1446**:156-160.
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44. Xu, Y., J. He, X. Wang, T. M. Lim and **Z. Gong** (2000) Asynchronous activation of 10 muscle specific protein (MSP) genes during zebrafish somitogenesis. *Dev. Dyn.* **219**:201-215.
45. Wang, X., H. Wan, V. Korzh and **Z. Gong** (2000) Application of an IRES bicistronic construct to trace the expression of exogenously introduced mRNA in zebrafish embryos. *Biotechniques* **29**:814-820.
46. Wang, H., T. Yan, J.T.T. Tan and **Z. Gong** (2000) A zebrafish vitellogenin gene (vg3) encodes a novel vitellogenin without a phosvitin domain and may represent a primitive vertebrate vitellogenin gene. *Gene* **256**: 303-310.
47. Gay, F., I. Anglade, **Z. Gong** and G. Salbert (2000) The LIM/homeodomain protein Islet-1 modulates estrogen receptor functions. *Mol. Endocrinol.* **14**:1627-48.

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48. Douglas, S.E., J.W. Gallant, **Z. Gong** and C. Hew (2001) Cloning and developmental expression of a family of pleurocidin-like antimicrobial peptides from winter flounder, *Pleuronectes americanus* (Walbaum). *Dev. Comp. Immunol.* **25**:137-147.
49. Sudha, P.M., S. Low, J. Kwang and **Z. Gong** (2001) Multiple tissue transformation in adult zebrafish by gene gun bombardment and muscular injection of naked DNA. *Marine Biotech.* **3**:119-125.
50. Wang, X., A. Emelyanov, I. Sleptova-Friedrich, V. Korzh, and **Z. Gong** (2001) Expression of two novel zebrafish Iroquois homologues (*ziro1* and *ziro5*) during devlopment of axial structures and central nervous system. *Mech. Dev.* **105**:191-195.
51. Chong, S.W., A. Emelyanov, **Z. Gong**, and V. Korzh (2001) Expression pattern of two zebrafish genes, *cxcr4a* and *cxcr4b*. *Mech. Dev.* **109**:347-354.
52. Wang, X., L.T. Chu, J. He, A. Emelyanov, V. Korzh and **Z. Gong** (2001) A novel zebrafish bHLH gene, neurogenin3, is expressed in the hypothalamus. *Gene* 275:47-55.

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54. Venugopal, T., V. Anathy, T.J. Pandian, **Z. Gong** and S. Mathavan (2002). Molecular cloing of growth hormone encoding cDNA of an Indian major carp, Labeo rohita and its expression in E.coli and zebrafish. *Gen. Comp. Endocrinol.* **125**: 236-247.
55. Wan, H., J. He, B. Ju, T. Yan, T.J. Lam and **Z. Gong** (2002) Generation of two-color transgenic zebrafish using the green and red fluorescent protein reporter genes, *gfp* and *rfp*. *Marine Biotech.* **4**: 146-154.
56. Zeng, S. and **Z. Gong**. (2002) EST analysis of expression profiles of zebrafish testis and ovary. *Gene* **294**: 45-53.
57. Wang, X., V. Korzh and **Z. Gong**. (2002) The functional specificity of NeuroD protein is defined by a single amino acid residue (N11) in the basic domain. *FEBS Lett.* **520**: 139-144.

58. Tan, C.H., T.C. Lee, S.D. Weeraratne, V. Korzh, T.M. Lim and **Z. Gong** (2002) *Ziwi*, the zebrafish homologue of the Drosophila *piwi*: co-expression with vasa at the embryonic genital ridge and gonad-specific expression in the adults. *Mech. Dev.* **119**:S221-S2244 (Also in *Gene Exp. Pattern* **2**:257-260).
59. Lam, S.H., H.L. Chua, **Z. Gong,** Z. Wen, T.J. Lam and Y.M. Sin (2002) Morphologic transformation of the thymus in developing zebrafish. *Dev. Dyn.* **225**:87-94.

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61. Irwin, D.M. and **Z. Gong** (2003) Molecular evolution of vertebrate goose-type lysozyme genes. *J. Mol. Evol.* 56:234-242.
62. Wang, X., A. Emelyanov, V. Korzh and **Z. Gong** (2003) Zebrafish atonal homologue *zath3* is expressed during both neurogenesis and gliogenesis. *Dev Dyn.* **227**:587-92.
63. **Gong, Z.**, H. Wan, T.L. Tay, H. Wang, M. Chen and T. Yan (2003) Development of transgenic fish for ornamental and bioreactor by strong expression of fluorescent proteins in the skeletal muscle. *Biochem. Biophys. Res Comm.* 388:58-63.

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65. Tong, Y., T. Shan, Y.K. Poh, T. Yan, H. Wang, S.H. Lam and **Z. Gong** (2004) Molecular cloning of zebrafish and medaka vitellogenin genes and comparison of their expression in response to 17β-estradiol (E2). *Gene* **328**:25-36.
66. Mudumana, S.P., H. Wan, M. Singh, V. Korzh and **Z. Gong** (2004) Expression analyses of zebrafish *transferrin*, *ifabp* and *elastaseB* mRNAs as differentiation markers for the three major endodermal organs: liver, intestine and exocrine pancreas. *Dev. Dyn.* **230**:165-173.
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71. Lam, S.H., Y.M. Sin, **Z. Gong** and T.J. Lam (2005) Effects of thyroid hormone on the development of immune system in zebrafish. *Gen. Comp. Endocrinol.* 142:325-335.
72. Wang, H., J.T.T. Tan, A. Emelyanov, V. Korzh and **Z. Gong** (2005) hepatic and extrahapatic expression of vitellogenin genes in the zebrafish *Danio rerio*. *Gene* 356:91-100.
73. Zeng, Z., X. Liu, S. Seebah and **Z. Gong** (2005) Faithful expression of living color reporter genes in transgenic medaka under two tissue-specific zebrafish promoters. *Dev. Dyn.* 234:387-392.
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80. Wan, H., S. Korzh, Z, Li, S.P. Mudumana, V. Korzh Y.-J. Jiang, S. Lin and **Z. Gong** (2006) Analyses of pancreas development by generation of *gfp* transgenic zebrafish using an exocrine pancreas-specific *elastaseA* gene promoter. *Exp. Cell Res.* 312:1526-1539.
81. Liu, X., H. Wang and **Z. Gong** (2006) Tandem-repeated Zebrafish *zp3* Genes Possess Oocyte-specific Promoters and Are Insensitive to Estrogen Induction. *Biology of Reproduction* 74:1016-1025.
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85. Song, J., H.J. Kim, **Z. Gong**, N.-A. Liu and S. Lin (2007) *Vhnf1* acts downstream of Bmp, Fgf, and RA signals to regulate endocrine beta cell development in zebrafish. *Dev. Biol.* 303:561-575.
86. Zhu, S., L. Liu, V. Korzh, **Z. Gong** and B.C. Low (2007) RhoA prevents apoptosis during zebrafish embryogenesis through activation of Mek/Erk pathway. Oncogene. 27:1580-1589..
87. Li. Z., V. Korzh and **Z. Gong** (2007) Localized rbp4 expression in the yolk syncytial layer plays a role in yolk cell extension and early liver development. BMC Dev Biol. 7:117 [Epub ahead of print].
88. Wu, Y.L., X. Pan, H. Wang, P.W. Kee, S.P. Mudumana and **Z. Gong** (2007) Development of a heat shock inducible gfp transgenic zebrafish line by using the zebrafish hsp27 promoter. Gene. 408:85-94.
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92. Pan X, Zhan H, Gong Z. (2008) Ornamental Expression of Red Fluorescent Protein in Transgenic Founders of White Skirt Tetra (Gymnocorymbus ternetzi). *Mar Biotechnol* 10:497-501.
93. Asharani, P.V., Y.L. Wu, Z. Gong and S. Valiyaveettil (2008) Toxicity of silver nonoparticles in zebrafish model. *Nonotechnology* 19:255102.
94. Asharani, P.V., N.G.B. Serina, M.H. Numawati, Y.L. Wu, Z. Gong and S. Valiyaveettil (2008) Impact of multi-walled carbon nanotubes on aquatic species. *J. Nonosci. Nonotech.* 8: 3603-3609.
95. Lam, SH, Mathavan S, Tong Y, Li H, Karuturi RKM, Wu YL, Vega VB, Liu ET and Gong Z. (2008) Zebrafish Whole-Adult-Organism Chemogenomics for Large-Scale Predictive and Discovery Chemical Biology. PLOS Genetics. 4:e1000121.
96. Liu, L., S. Zhu, Z. Gong and B.C. Low (2008) K-ras/PI3K-Akt signaling is essential for zebrafish hematopoiesis and angiogenesis. *Plos One* 3:e2850.
97. Liu, X., Z. Li, A. Emelyanov, S. Parinov and Z. Gong (2008) Generation of oocyte-specifically expressed cre transgenic zebrafish for female germline excision of loxP-flanked transgene. *Dev. Dyn.* In press.
98. Li, Z., C. Wen, J. Peng, V. Korzh and Z. Gong (2008) Generation of living color transgenic zebrafish to trace somatostatin-expressing cells and endocrine pancreas organization. *Differentiation* In press.

B. Invited Reviews, Monographs and Book Chapters

1. Hew, C.L., S.J. Du, **Z. Gong**, G.L. Fletcher, and P.L. Davies (1991) Biotechnology in aquatic sciences: improved freezing tolerance and enhanced growth in Atlantic salmon by gene transfer. *Bull. Insti. Zool., Academia Sinica* (Taipei). 16:341-356.
2. Hew, C.L., and **Z. Gong** (1992) Transgenic fish: a new technology for fish biology and aquaculture. *Biology International* **24**:2-10.
3. Du, S.J., **Z. Gong**, G.L. Fletcher, M.A. Shears, and C.L.Hew (1992) Growth hormone gene transfer in atlantic salmon: use of fish antifreeze/growth hormone chimeric gene constructs and application of polymerase chain reaction. in "Transgenic Fish", C.L. Hew and G.L. Fletcher, eds. pp.176-189.
4. **Gong, Z.** and C.L. Hew (1993) Promoter analysis of fish antifreeze protein genes. *Biochemistry and Molecular Biology of Fishes*, volume 2, (P.W. Hochachka and T.P. Mommsen, eds) pp.307-324.
5. Xiong, F., R. Chin, **Z. Gong**. K. Suzuki. R. Kitching, S. Majumdar-Sonnylal, H.P. Elsholtz and C.L. Hew (1993) Control of salmon pituitary hormone gene expression. *Fish Physiol. Biochem.* **11**:63-70.
6. Hew, C.L., S.J. Du, **Z. Gong**, P.L. Davies, S.Y. Gauthier, M.A.Shears, M.J. King and G.L. Fletcher. (1993) Transgenic salmon with enhanced growth and freeze resistance. *Biology International* **28**:87-94.
7. **Gong, Z.**, and C.L. Hew (1995) Transgenic fish in aquaculture and developmental biology. *Current Topic Dev. Biol.* **30**:177-214.
8. **Gong, Z.**, S.J. Du, G.L. Fletcher, P.L. Davies and C.L. Hew (1995) Application of transgenic fish technology in aquaculture. Proceedings of the International Symposium on Biotechnology Applications in Aquaculture, C.-M. Kuo, J.-L. Wu, and P.-P. Hwang, eds. pp.41-54.
9. Hew, C.L., **Z. Gong**, S.J. Du, M.A. Shears, M.J. King, G.L. Fletcher, P.L. Davies, and R. Saunders (1998) Use of the fish antifreeze protein gene promoter in the production of growth hormone-transgenic salmon with enhanced growth performance. In: Biotechnology in Agriculture. A. Altman (ed) Marcel Decker, Inc., NY. pp.549-561.
10. Fletcher, G.L., S.V. Goddard, P.L. Davies, **Z. Gong**, K.V. Ewart and C.L. Hew (1998) New insights into fish antifreeze proteins: physilogical significance and molecular regulation. In: Cold Ocean Physiology. H.O. Portner and R.C. Playle (eds). Cambridge University Press.
11. **Gong, Z.** (1999) Zebrafish expressed sequence tags and their applications. *Methods Cell Biology* **60**:213-233.
12. **Gong, Z.**, P.M. Sudha, B. Ju, Y. Xu, J. He, H. Wang, S. Zeng, H. Wan, X. Wang and T. Yan (1999) Applications of the transgenic technique to fish and shrimps. *J. Ocean Univer. Qingdao* 29:649-657.
13. Anglade, I, S. Planchot, **Z. Gong**, C.L. Hew, O. Kah and G. Salbert (1998) Distribution of islet-expressing cells in the forebrain and pituitary of the rainbow trout. *Ann NY Acad. Sci.* **839**:420-421.
14. **Gong, Z.**, B. Ju and H. Wan (2001) *Green fluorescent protein* (*GFP*) transgenic fish and their applications. *Genetica* **111**:213-225.

15. Melamed, P., **Z. Gong**, G.L. Fletcher, and C.L. Hew, (2002) The impact of biotechnology in aquaculture. *Aquaculture* **204**:255-269
16. **Gong, Z.**, H. Wan, B. Ju, J. He, X. Wang and T. Yan (2002) Generation of living color transgenic zebrafish. In: Aqua Genome: Steps toward a Great Future. N. Shimizu, T. Aoki, I Hirono, F. Takashima (eds). Pp.329-339.
17. **Gong, Z.**, H. Wan, M. Chen and T. Yan (2002) Applications of transgenic technology in ornamental fish. *Fisheries Sci.* **68**:1063-1066.
18. **Gong, Z.**, Y.L. Wu, S.P. Mudumana and S. Lin (2004) Transgenic fish for developmental biology studies. In: Fish Development and Genetics: the zebrafish and medaka model. Z. Gong and V. Korzh (eds). World Scientific. Pp.476-516.
19. Low, B.C. and **Z. Gong** (2005) Reporter Gene System: Green Fluorescent Protein. In: Encyclopedia of Molecular Cell Biology and Molecular Medicine. 2nd Edition. R.A. Meyers (ed). Wiley-VCH. Volume 12: 215-248.
20. **Gong, Z.**, N. Maclean, R.H. Devlin, R. Martinez, O. Omitogun and M. P. Estrada (2007) Chapter 4: Gene Construct and Expression: Information Relevant for Risk Assessment and Management. In: Environmental Risk Assessment of Genetically Modified Organisms: Methodologies for Transgenic Fish (eds; A. Kapuscinski, S. Li and K. Hayes).
21. Kapuscinski, A., G. Dana, K. Hayes, S. Li, K. Nelson, Y. K. Nam, **Z. Gong**, R. Devlin, G. Mair and W. Senanan (2007) Chapter 10: Risk Assessment of Transgenic Fish: Synthesis and Conclusions. In: Environmental Risk Assessment of Genetically Modified Organisms: Methodologies for Transgenic Fish (eds; A. Kapuscinski, S. Li and K. Hayes).

C. Book

1. **Gong, Z.** and V. Korzh (2004) Fish Development and Genetics: the zebrafish and medaka models. World Scientific. PP. 675.

4. Research Grants:

University research grants:

1. PI. Academic Research Fund, $223,335, 1995-1998: Developmental regulation and functional analysis of a family of LIM domain homeobox genes in zebrafish.

2. PI, Academic Research Fund, $70,330, 1995-1997: Identification of surface antigens in *Ichthyophthirius multifiliis* and the development of fish vaccine.

3. PI. Academic Research Fund, $114,550, 1996-1999: Generation of novel varieties of ornamental fish by transgenic expression of green fluorescent protein (GFP).

4. Collaborator. Academic Research Fund, $151,200, 1996-1999: Sex differentiation in fish (PI, A/P Tan Choong Huat).

5. PI. Academic Research Fund, $245,000, 1998-2002: Molecular dissection of neurogenic pathway in zebrafish.

6. PI, Academic Research Fund, $74,550, 1999 -2003: Production of fluorescent transgenic ornamental fish.

7. PI, Academic Research Fund, $175,395, 1999 - 2003: Transgenic expression of spider silk protein genes in the silkworm, *Bombyx mori*.

8. Collaborator. Academic Research Fund, $73,250, 2000-2003: Gonadogenesis during early development of fish embryos. (PI. A/P Tan Choong Huat)

9. Collaborator, Academic Research Fund, $86,085, 2000-2003: Development of the immue system in fish larvae and its possible control by thyroid hormones. (PIs: Prof. Lam Toong Jin and A/P Sin Yoke Min)

10. Collaborator, Academic Research Fund, $63,069, 2001-2003: Sextual behaviour, reproduction and viability of fluorescent transgenic zebrafish. (PI, Dr. Li Daiqin)

Research projects funded by government agencies or industry:

1. PI, ENV/PUB/NSTB Newater project for fish test and research 1999-2002 (Phase I): budgeted, $620,000; secured, $461,850. Fish test in Water Quality Program under National Water Reclamation (NEWater).

2. Co-PI, NSTB grant 2001-2004, Establishment of a lab of excellence in aquatic and marine biotechnology (LEAMB) (S$1.5M, shared with four other groups, PI, Prof. Hew Choy Leong)

3. Co-PI of toxicogenomics in BFIG-II (Biosensor Focus Group, Phase II). Funded by NSTB/NUS (2001-2003). (PI, Prof. P. Gopalakrishnakone)

4. PI, ENV/PUB/NSTB Newater project, $228,000; 2002 -2003 (extension): Fish test in Water Quality Program under National Water Reclamation (NEWater).

5. PI, (co-PIs: Drs. B.C. Low, V. Korzh, E. Liu, S. Mathavan, R. Ge, W.K. Chan, T.P. Loh and R. Shoba) BMRC (Biomedical Research Council of Singapore) co-operative grant 2002-2007, $5,354,970. Molecular mechanisms of liver development and hepatocarcinogenesis: the zebrafish model.

6. Co-PI (PI, Dr. Jiang Yun-Jin) A*STAR BMRC-JDRF GRANT: Notch signalling and control of stem cell differentiation in zebrafish pancreas (04/1/50/22/295), 2004, S$248,750 for 2 years (11/12/04-10/12/06).

7. PI (co-PI, Drs. Ong Chone Nam, Lam Siew Hong, Xie Rongjing, Zhang Lifeng, S. Mathavan, E. Liu) EWI (Environment and Water Industrial Council), 2007-2010. $1,699,000. Use of Small Aquarium Fish to Develop Biological Monitoring Systems for Water Quality and Security.

8. PI (collaborators: V. Korzh, A. Ip, Hew CL) MOE Tier 2. 2007-2010. $632,600. Molecular Characterization of Fish Swimbladder Development: Implications of the Origin and Evolution of Lung in Tetrapods

5. Membership of institutional, national or international scientific advisory boards

1. Board member, IUBS-RBA (International Union of Biological Sciences--Reproductive Biology in Aquaculture), since June 1997.
2. Elected member of Asia-Pacific IMBN (International Molecular Biology Network), since Jan. 2001.
3. Scientific Advisory Board of Yorktown Technologies, USA, the GloFish company. My role is as an inventor of GloFish^{TM} to provide expert opinion on development of new varieties of transgenic GloFish^{TM} and to evaluate their environmental impact for USA government regulatory purpose. Since 2002
4. Committee member of International Society of Aquatic Genomics, since 2003.
5. Invited member of GMAC for reviewing transgenic fish, Singapore. 2003
6. Invited member of Advisary Board of Science Center, Singapore. Since 2003

6. Membership of Editorial Boards

1. Co-editor of Molecular Aspects of Fish and Marine Biology, World Scientific (since 1999). So far, four volumes of monographs have been published.

2. **Assistant Editor of *Journal of Fish Biology*** (since Jan 2006). My role is to conduct review and revision of manuscripts on fish molecular biology and to make recommendation of acceptance or rejection of the manuscripts.

10. Invited speakers at conference/symposia

Prior to NUS appointment (1993-1995)

1. Invited Speaker, "The LIM domain homeobox gene isl-1 in salmon". Taniguchi Symposium on Developmental Biology V: Gene Regulation in Development of Aquatic Animals. Qingdao, China. April 1993.
2. Invited Instructor of IUBS workshop on Reproductive Biology and Agriculture, Qindao, China, April 1993
3. Invited speaker, "From gene tagging to transciption factors". IUBS Symposium: Advances in the Molecular Endocrinology of Fish. Toronto. May 1993.
4. Invited speaker, "Application of transgenic fish technology in aquaculture" . International Symposium on Biotechnology Applications in Aquaculture, Taipei, Taiwan. Dec. 1994.
5. Invited speaker, "A family of novel LIM homeodomain proteins from teleosts: molecular characterization, expression analysis and DNA binding properties". Workshop on LIM Proteins and the LIM Domain, Bischenberg, France, May 1995.

Representing NUS (since 1996)

1996

6. Invited Plenary Keynote Speaker, "Transgenic fish and marine biotechnology" Asia-Pacific Conference on Science and Management of Coastal Environment, Hong Kong, June 25-28, 1996.

1997

7. Invited Speaker, "Sequence tag project in the zebrafish" in Current Advances in Defining the Zebrafish Genome, Boston, MA, U.S.A. Feb. 2-4, 1997.
8. Invited Session Chair and Speaker, "Zebrafish neuroD, a potential upstream gene of the neuroendocrine transcription factor Isl-1". 2nd IUBS Toronto Symposium "Advances in the Molecular Endocrinology of Fish" May 16-19, 1997, Toronto, Canada.
9. Invited Session Chair and Speaker, "Massive cloning of zebrafish genes and their applications". 7th SCBA International Symposium. July 6-11, 1997, Toronto, Canada.

1998

10. Invited Keynote Speech, "Massive cloning of fish genes and their applications in transgenic fish". International Symposium on Progress and Prospect of Marine Biotechnology (ISPPMB'98), Oct. 6-9, 1998, Qingdao, China.
11. Invited Speaker, "Application of transgenic techniques in fish and shrimp diseases" UNESCO workshop on shrimp disease, Oct. 9-14, 1998, Qingdao, China.

1999

12. Invited Session Chair and Speaker, "From zebrafish EST clones to transgenic ornamental fish". Aquarama'99: World Conference on Ornamental Fish Aquaculture. June 3-6, 1999, Singapore.

2000

13. Invited Plenary Keynote Speaker, "Application of transgenic technology in aquaculture and developmental biology". IMBC 2000: International Marine Biotechnology Conference. Sept. 28-Oct. 4, 2000, Townsville, Austrilia.
14. Invited Principal Speaker, "Generation of living color transgenic zebrafish". International Symposium: A Step Toward the Great Future of Aquatic Genomics. Nov. 10-12, 2000, Tokyo, Japan.
15. Invited Speaker. "Application of transgenic technology in aquaculture". 7th Pacific Rim Biotechnology Conference and Bioexpo 2000. Nov. 12-16, Vancouver, Canada.
16. Invited Speaker. "Generation of living color transgenic zebrafish" International Symposium on Marine Biotechnology, Dec. 6-8, 2000, Qindao, China.

2001

17. Invited Speaker, "Generation of living color transgenic zebrafish". International Conference on Advanced Technologies in Fisheries and Marine Sciences. Feb. 2-4, 2001, Tamil Nadu, India.
18. Invited Speaker, "Generation of living color transgenic ornamental fish". Aquarama: 2nd World Conference on Ornamental Fish Aquaculture "Modern Technology for the Future". May 31-June 2, 2001, Singapore.
19. Invited Speaker, "Applications of transgenic technology in ornamental fish". 70th Anniversary of the Japanese Society of Fisheries Science: International Commemorative Symposium. Oct. 1-5, 2001. Yokohama, Japan.
20. Invited Speaker and session chair, "Generation of rainbow color transgenic zebrafish". The Sixth Asian Fisheries Forum. Nov. 25-30, 2001, Kaohsing, Taiwan. (unable attend due to an urgent mission in the national NEWater project).

2002

21. Invited Speaker. "Generation of rainbow color transgenic zebrafish" World Aquculture 2002. April 23-27, 2002. Beijing, China. (unable to attend due to teaching duty, but the paper was presented by my student, Mr. Wan Haiyan, on my behalf).

2003

22. Invited Speaker, "Transgenic fish technology" 7th International Symposium on Reproductive Physiology of Fish. May 18-23, 2003, Mie, Japan. (unable to attend because of conduct of re-sit examination)
23. Invited Speaker, "Promises of transgenic fish biotechnology" Seminar on current Advances in Biotechnology. June 6, 2003. Jakarta, Indonesia.
24. **Invited Speaker, "Application of transgenic technology to ornamental fish" XIX International Congress of Genetics. July 6-11, 2003. Melbourne, Australia.**

Our research was highlighted in the Congress' daily briefing. Following my report I the Congress, I was interviewed by many world-wide media including *Scientific America, New Scientists* and many Australian media and our research story had been published in many Australia newspapers and radios during the Congress.

25. Invited Speaker, "Transgenic fish for ornamentals and bioreactors" AusBiotech2003. Aug. 16-19, Adelaide, Australia.
26. Invited Keynote Speaker, "Transgenic fish for ornamentals and bioreactors". 6th International Marine Biotechnology Conference. Sept. 21-24, 2003. Chiba, Japan.

27. Invited Speaker, "Zebrafish DNA chip" 2nd Aquatic Genomic Conference, Sept. 25-27. 2003. Tokyo, Japan.
28. Invited Speaker, "transgenic fish" Biosafety Science of Genetically Engineered Organisms. Oct. 27-28, 2003. Chonburi, Thailand.
29. Invited Speaker, "Application of transgenic technology to ornamental fish" The 8th International Aquarium Fish & Accessories Exhibition & Conference, Oct 30 – Nov. 2, 2003, Singapore.

2004

30. Invited Speaker, "Transgenic fish for ornamentals, bioreactors, vaccines and biomonitoring". World Aquaculture 2004, March 1-4, 2004, Honolulu, USA.
31. Invited Public Lecturer, "Future of transgenic fish". Singapore International Fish Show. March 12-15, 2004, Singapore.
32. Invited speaker, on aquaculture biotechnology. Indo-Singapore Joint Workshop on Aquaculture and Marine Biotechnology, April 22-24, Kochi, India. (did not attend because of university exam).
33. Invited Speaker, Exploration and Application of Marine Gene Resources. May 18-20. Beijing, China. (did not accept)
34. Invited Speaker, "Zebrafish model in human diseases" Sir Edward Youde Memorial Fund Postgraduate Conference 2004-Model Organism Research and Human Diseases. June 14-15, 2004, Hong Kong.
35. Invited Speaker, "China International Recreation Fisheries and Aquaria 2004 Conference" Sept. 9-12, 2004, Guangzhou, China. (did not accept because of teaching duty).
36. Invited Speaker, "Of fish and chips: Genome-wide expression profiling studies using a zebrafish DNA chip". Fish Genetics and Development. Oct. 11-14, 2004. Wuhan, China.
37. Invited Speaker, "Genome-wide expression profiling studies using a zebrafish DNA chip" Asian/Oceanian fish meeting, Nov. 15-16, 2004, RIKEN, Kobe, Japan.
38. Invited Speaker, Sixth Asia-Pacific Marine Biotechnology Conference: Unique Processes and Novel Products, Nov. 28-Dec. 2, 2004, Zhoushan, China (did not accept)
39. Invited Speaker, 1. "DNA microarray technology in fish"; 2. "Transgenic fish technology". Workshop on "Genomics and Its Related Techniques Applied to Aquatic Organisms". Dec. 17-22, 2004, Shanghai, China.
40. Invited Keynote Speaker, "Application of transgenic fish technology". 1st COE International Symposium on "Potential and Perspective of Marine Bio-Manipulation" Feb. 26-27, 2004, Sapporo, Japan

2005

41. Invited Speaker, "A fish model in environmental monitoring and cancer research: the DNA microarray approach", Workshop on Pearl Oyster Genome and Application. March 23, 2005, Haikou, China,
42. Invited Speaker, International Symposium on Genetically Modified Organisms. Sept 2-3, 2005, Jeju City, Korea (did not make it because of sabbatical leave)
43. Invited Participant and Chapter Leader. United Nations Environmental Programme STAP (Scientific and Technical Advisory Panel) book writing workshop on the

environmental risk assessment of transgenic fish. 17-21 October 2005, Penang, Malaysia.

44. Invited Speaker. International Congress "Biotechnology Havana 2005", Nov. 27-Dec. 2, 2005, Havana, Cuba. (did not make it because of sabbatical leave)

2006

45. Invited Speaker, "Promising applications of transgenic fish technology in aquaculture, environmental monitoring and cancer biology". 2nd Norwegian Transgenic Animal Forum, March 23-24, 2006, Vikersund, Norway.

2007

46. Invited Speaker, "Asian biotech aquaculture and the perspectives of Asian governments on sustainability" (topic given by the organizer) Aquaculture America '07. February 26 to March 2, 2007, San Antonio, Taxes, USA.
47. Invited Speaker, "Zebrafish as a human disease model" (tentative) Model Systems for Infectious Disease and Cancer in Zebrafish, Jul 16-18, 2007, Amsterdam, Holland.
48. Invited Speaker, "Applications of transgenic fish and fish DNA chips in environmental monitoring". International Symposium on Biotechnology in Agriculture. July 26-27, 2007. Qingdao, China.

Exhibit 2

Gene, 173 (1996) 89–98
 0378-1119/96/$15.00

GENE 09432

Green fluorescent protein marks skeletal muscle in murine cell lines and zebrafish *

(Transfection; *Danio rerio*; embryo; myosin light chain; vital dye)

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SUMMARY

The green fluorescent protein (GFP) acts as a vital dye upon the absorption of blue light. When the *gfp* gene is expressed in bacteria, flies or nematodes, green fluorescence can be directly observed in the living organism. We inserted the cDNA encoding this 238-amino-acid (aa) jellyfish protein into an expression vector containing the rat myosin light-chain enhancer (MLC-GFP) to evaluate its ability to serve as a muscle-specific marker. Transiently, as well as stably, transfected C2C12 cell lines produced high levels of GFP distributed homogeneously throughout the cytoplasm and was not toxic through several cell passages. Expression of *MLC-GFP* was strictly muscle-specific, since Cos 7 fibroblasts transfected with *MLC-GFP* did not fluoresce. When GFP and βGal markers were compared, the GFP signal was visible in the cytoplasm of the living cell, whereas visualization of βGal required fixation and resulted in deformation of the cells. When the *MLC-GFP* construct was injected into zebrafish embryos, muscle-specific *gfp* expression was apparent within 24 h of development. *gfp* expression was never observed in non-muscle tissues using the *MLC-GFP* construct. Transgenic fish continued to express high levels of *gfp* in skeletal muscle at 1.5 months, demonstrating that GFP is an effective marker of muscle cells in vivo.

INTRODUCTION

The beautiful green bioluminescence emanating from the circular and radial food canals of the jellyfish *Aequoria victoria* is ultimately caused by the emission of green light from a cyclical amino-acid (aa) chromophore which is an integral part of the green fluorescent protein (GFP) (Prasher et al., 1992). When *gfp* cDNA was cloned and expressed in bacteria, *Caenorhabditis elegans* (Chalfie et al., 1994) or in *Drosophila melanogaster* (Wang et al., 1994), a green fluorescence (GF), with an emission peak at 509 nm, was directly visualized in vivo. Many reporter systems allow the quantitation and localization of proteins expressed within prokaryotic as well as eukaryotic cells. A distinct advantage of GFP is that its fluorescence depends only upon the presence of the protein (inducible at blue excitation wavelengths in the 360–420 nm range). Therefore, GFP is a marker whereby GF can be plotted

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Abbreviations: *A.*, *Aequoria*; aa, amino acid(s); βGal, β-galactosidase (product of gene *lacZ*); bp, base pair(s); *C.*, *Caenorhabditis*; CAT, chloramphenicol acetyltransferase; cDNA, DNA complementary to RNA; CMV, cytomegalovirus; DMEM, Dulbecco's modified Eagle's medium; dNTP, deoxyribonucleotide triphosphate; *D.*, *Drosophila*; FCS, fetal calf serum; FITC, fluoroisothiocyanate; GF, green fluorescence; GFP, GF protein; *gfp*, gene (DNA) encoding GFP; Hy, hygromycin; kb, kilobase(s) or 1000 bp; MLC, myosin light chain; *MLC*, gene encoding MLC; *MLCE*, *MLC* element; nt, nucleotide(s); PCR, polymerase chain reaction; *p*, promoter; p, plasmid; PolIk, Klenow (large) fragment of *E. coli* DNA polymerase I; ^{R}, resistant/resistance; S65T, $Ser^{65} \rightarrow Thr$; *UTR*, untranslated region(s); wt, wild type.

SSDI 0378-1119(95)00729-6

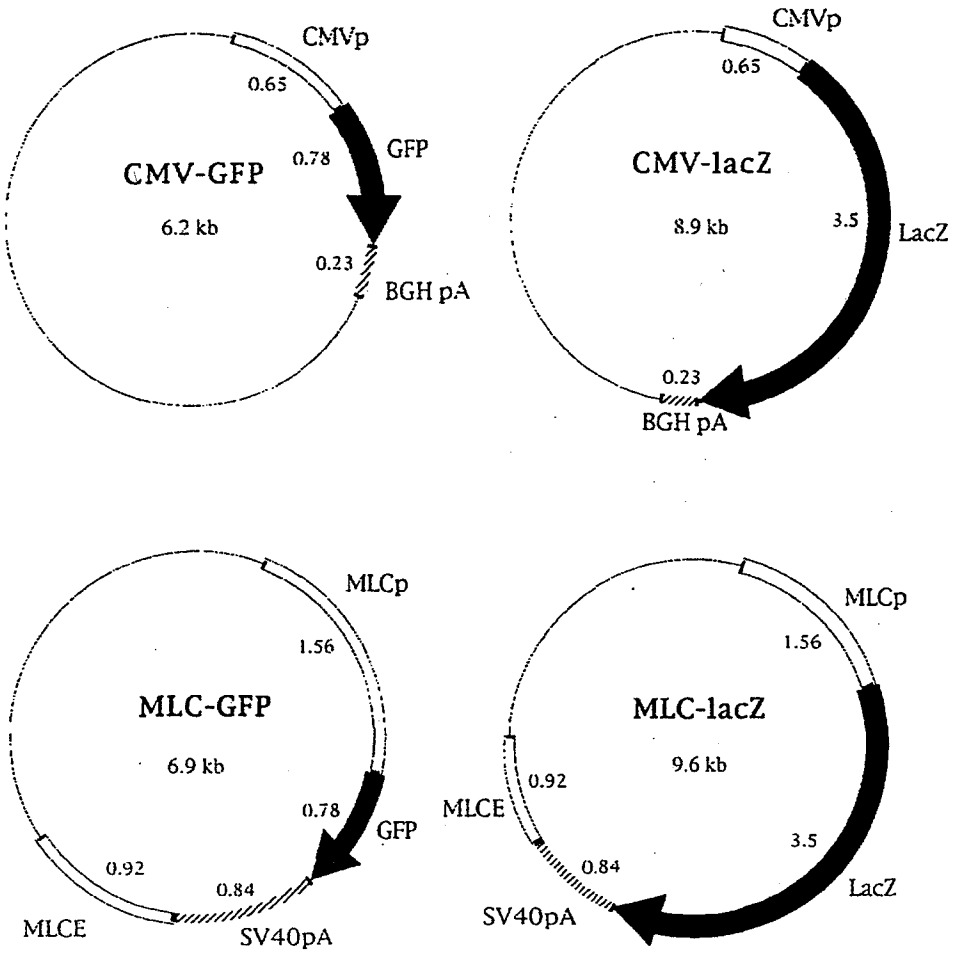
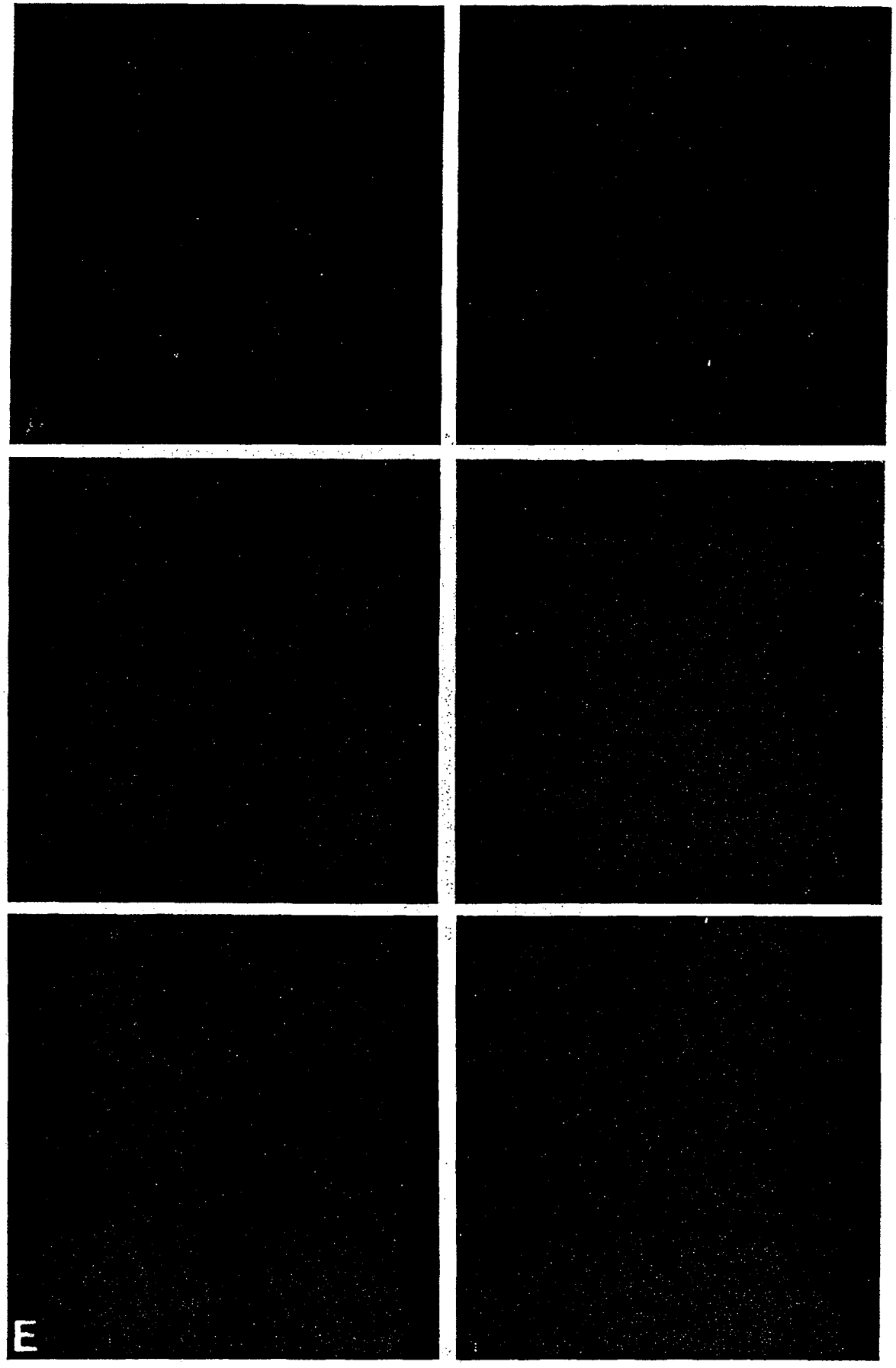


Fig. 1. The *gfp* plasmid expression vectors were derived from TU#65, a Bluescript (Stratagene, La Jolla, CA, USA) vector containing the full-length cDNA fragment from *A. victoria* (Chalfie et al., 1994). A rat *MLC* expression vector (Donoghue et al., 1988) with a unique *Hin*dIII site at the junction between the 1.5-kb *MLC* promoter and the 0.8-kb SV40 poly(A) sequence fused to the muscle specific *MLC* 0.9-kb enhancer was linearized, then blunt ended using PolIk and dNTPs (Ausubel et al., 1993). A micrococcal-nuclease-treated 0.78-kb *Kpn*I-*Eco*RI *gfp* fragment from TU#65 was subsequently ligated into the *MLC* vector to create *MLC-GFP*. CMV vectors were constructed in pcDNA3 (Invitrogen). The *Kpn*I-*Eco*RI *gfp* cDNA was directionally cloned into the corresponding sites of the pcDNA3 polylinker (*CMV-GFP*). The *lacZ* expression vectors were constructed with a 3.5-kb *Not*I fragment of an SV40 *lacZ* vector (pSVβ, MacGregor and Caskey, 1989) inserted into the *Not*I site of pcDNA3 to yield *CMV-lacZ*, or blunt end ligated into the *MLC-GFP* vector described above, to generate *MLC-lacZ*. A serine within the chromophore was mutated to a Thr (TCT to ACT) by a modified PCR mutagenesis (Innis et al., 1990). The mutation was confirmed by DNA sequencing and the mutated *gfp* was cloned into pcDNA3 for comparison to wt.

Fig. 2. Applications of GFP. (**A**) Live cultures of Cos 7 cells transfected with *CMV-GFP* (10× lens). (**B**) Background fluorescence of untransfected Cos 7 cells (10× lens). (**C**) Bright field photograph of growing Cos 7 fibroblasts. (**D**) Cos 7 cells transfected with *CMV-lacZ* then fixed after 48 h in culture (20× lens). βGal activity was visualized as previously described (Ausubel et al., 1993). (**E**) Live Cos 7 cell cultures transfected with *CMV-GFP* containing the S65T mutation (25× water immersion lens). (**F**) Live Cos 7 cells transfected with wt *CMV-GFP* photographed under the same conditions as for **E**. **Methods:** The indicated plasmids were CsCl gradient purified then transiently transfected into Cos 7 fibroblast cell lines. 60 mm culture dishes containing 5×10^5 cells were incubated for 8 h with 3 μg DNA/10 μl lipofectamine (Gibco BRL)/3 ml DMEM. The transfection media was removed and replaced with culture medium (DMEM with 10% FCS/100 units streptomycin/100 μg penicillin/ml) for 14–20 h. GF was visualized using an Optiquip model #1500 power supply with a Xe/Hg light source set at 200 W (Hg lines). Photographs were taken on 400 speed film through a Zeiss axioplan microscope. The filter set was either a Zeiss #10 (FITC) or a customized set which eliminates some of the green autofluorescence (Chroma standard GFP set).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over time in living cells or transparent organisms with no addition of exogenous substrates. Bacteria engineered with a T7 RNA polymerase promoter generate high levels of GFP (Chalfie et al., 1994; Inouye et al., 1994) that can be aerobically cultured without loss of GF or toxicity to the cells. In *C. elegans*, the β-tubulin promoter *mec-7* produces bright green touch-receptor neurons that can be observed continuously during development in the same animal (Chalfie et al., 1994). When a maternally-derived GFP fusion protein is expressed in transgenic *D. melanogaster*, the fluorescent oocytes are resistant to photobleaching, are homogeneously labeled and can be directly observed with the confocal microscope in living, developing egg chambers (Wang et al., 1994). Other applications for GFP as a vital dye are just beginning to be realized.

Because GFP is a relatively small protein that fluoresces without the need for additional accessory molecules we evaluated its potential for tissue-specific expression during muscle development. The availability of muscle-specific control regions with known developmental profiles, such as the promoter and enhancer from the rat myosin light chain 1/3 locus, provide the opportunity to produce GFP-marked muscle cells which can be followed over time. The results of this report indicate that GF is readily visible in murine C2C12 muscle cells as transient and stable transfectants. Skeletal muscle fibers in transient transgenic zebrafish embryos and adults can be marked with GFP and observed with a fluorescent microscope in living animals. Expression is tissue specific, durable over time and non-toxic to the cell types we have studied. Muscle-specific GFP markers and different stage-specific control regions will provide powerful new tools for the study of zebrafish muscle development.

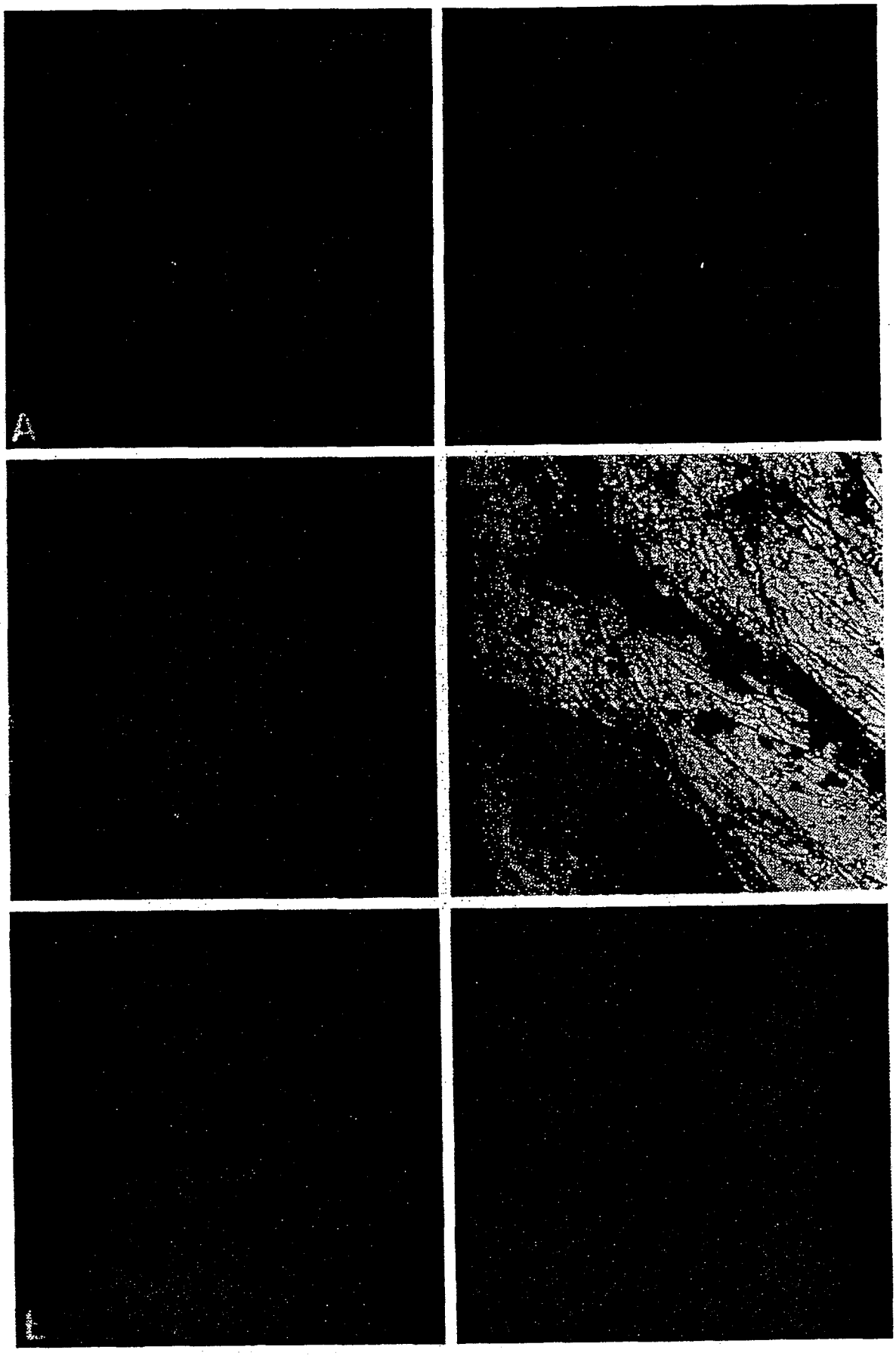
RESULTS AND DISCUSSION

(a) GFP is expressed transiently in Cos 7 fibroblasts and C2C12 myotubes

We determined if GFP could be used as a vital dye in transiently transfected cell lines by comparison with GFP and βGal signals. We placed the *gfp* cDNA from plasmid TU#65 (Chalfie et al., 1994) into two plasmids, one that was designed to function only in muscle cells (*MLC-GFP*) and one that could potentially be expressed in a variety of cell types (*CMV-GFP*). *MLC-GFP* (Fig. 1) is a pUC18-based myosin light chain vector consisting of 1.56 kb of the *MLC1* promoter, 0.84 kb of the small intron and polyadenylation site of the SV40 T antigen and 0.92 kb of the *MLC* enhancer (Donoghue et al., 1988). In transgenic mice, the *MLC* enhancer was sufficient to activate high levels of developmentally regulated gene expression from the *MLC1* promoter (Rosenthal et al., 1989). Further analysis of transgenic fetal and neonatal mice revealed that graded synthesis of CAT produced from this construct is established during the initial stages of somitogenesis and is maintained during subsequent maturation of somitic derivatives, persisting only in adult intercostal and intervertebral muscle groups (Donoghue et al., 1991; Grieshammer et al., 1992). In this study, the *MLC-GFP* construct lacked 5′ and 3′ *UTR* of *gfp*. The native jellyfish ATG, which is not a Kozak consensus sequence, was used for translation. For purposes of comparison, we inserted a *lacZ* reporter into the same site of a parallel *MLC* vector. We also generated *gfp* and *lacZ* mammalian expression vectors using the Invitrogen pcDNA3 plasmid (Fig. 1). This construct drives high-level stable and transient expression of genes inserted next to a *CMV* promoter.

When Cos 7 cells were transfected with *CMV-GFP*, bright GF was readily observable at 24 h post-transfection with a 10× lens using the standard GFP filter set from Chroma (Fig. 2A). Because this construct allows for episomal replication in Cos 7 cells which latently express the SV40 large T antigen, large amounts of GFP protein become concentrated in the cytoplasm. Positive cells were visualized without changing the media or perturbing the culture. Green auto-fluorescing dead cells which interfered with the GFP signal were removed by washing with phosphate buffered saline. Any remaining dead cells fluoresced under both GFP and Texas red filter sets, whereas the GF from GFP-positive cells was visible only with a GFP or FITC (fluoroisothiocyanate)

Fig. 3. GF of myotubes. (A) Live cultures of C2C12 cells transfected with *MLC-GFP* (25× lens, water immersion). (B) Background fluorescence of untransfected myotubes (25×). (C) Bright field image of myotubes in culture (25×). (D) C2C12 cells transfected with *MLC-lacZ* and fixed (20× lens). Panels E and F: two separate isolates of stable C2C12 myotubes. **Methods:** C2C12 myoblasts were seeded at 5×10^5 cells/60 mm dish in growth medium (DMEM/20% FCS/100 units streptomycin/100 μg penicillin/ml). Each lipofectamine transfection contained 20 μl lipofectamine and 5 μg DNA. The cells were incubated in growth media for 24 h, then differentiated to myotubes in low serum with DMEM/2% horse serum for an additional 48 h. *MLC-GFP* stable C2C12 cell lines were generated using a co-transfected Hy^R vector (p3′SS, Stratagene). 100-mm dishes were seeded with 0.1×10^6 myoblasts and lipofectamine-transfected using 15 μg of *MLC-GFP* and 1 μg of p3′SS in 6 ml of DMEM containing 30 μl lipofectamine. After 8 h in transfection media, cells were washed and replaced with growth media for 16 h. We generated a killing curve using Hy concentrations ranging from 200 to 800 μg/ml in growth media. We conclude that 600 μg Hy/ml is optimal for selection in C2C12 cells. The cells were incubated in selection media for one week and single colonies cloned and expanded. A portion of each clone was grown in low serum and tested for green fluorescence. Four of six Hy resistant lines resulted in bright green fluorescence upon differentiation into myotubes.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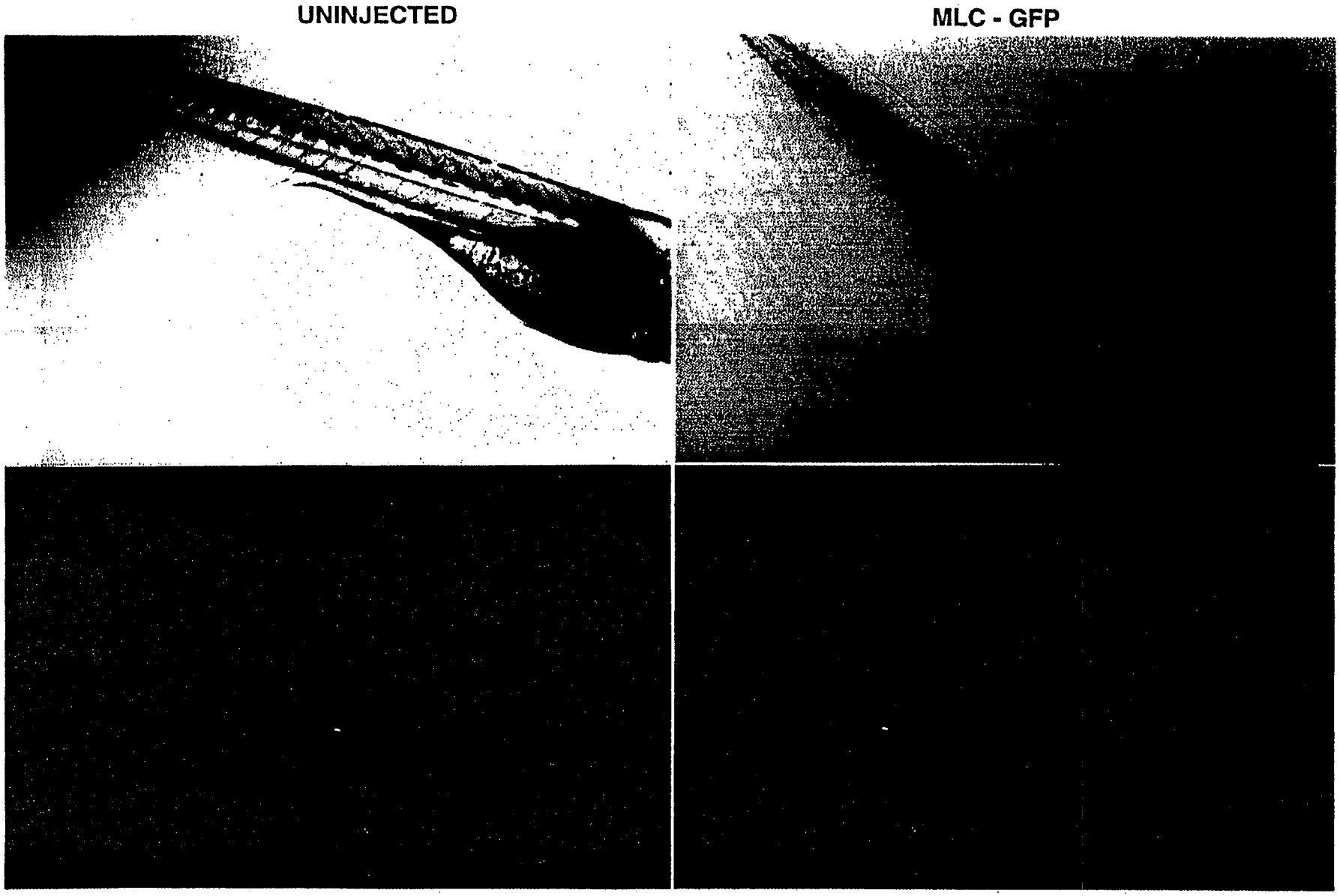

Fig. 4. Bright-field photograph and fluorescence image (below) of the same 36 h uninjected embryo (left) (pale green auto-fluorescence accumulated in the yolk sac) and of a 36 h *MLC-GFP* injected zebrafish (right). **Methods:** Zebrafish (*Danio rerio*) were mated and freshly fertilized eggs dechorionated with 5 μg pronase/ml. Embryos at the 2-, 4- or 8-cell stage were subsequently injected with 50 ng/μl of *MLC-GFP* or *CMV-GFP* plasmid DNA and 0.1% phenol red (Gibco BRL) as a marker. After 24 h of development in embryo media on agarose-coated culture dishes (Westerfield, 1993), the GFP was first visibly expressed in embryonic skeletal muscle fibers. All photographs were taken with a 5× lens. Fish were maintained in the system as previously described (Solnica-Krezel et al., 1994). For photography, zebrafish embryos were placed in a depression slide, anesthetized and positioned in methyl cellulose. Once revived in egg water, embryos were returned to the system.

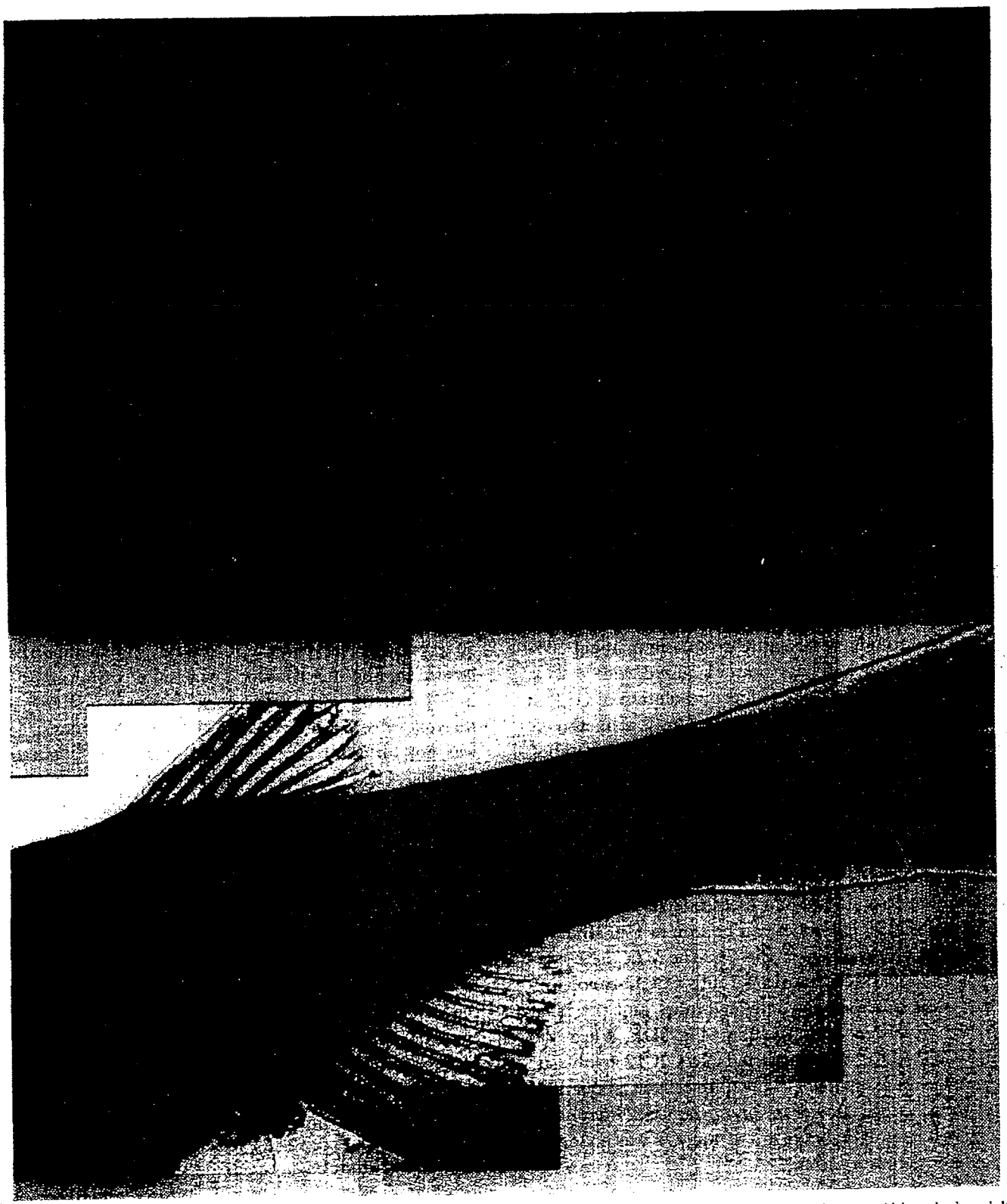

Fig. 5. Fluorescence (top) and bright field (bottom) photograph of a zebrafish at 1.5 months of development using a 5× lens Although the adult skeleton has formed and green auto-fluorescence accumulates in the swim bladder, this transiently transgenic zebrafish was still transparent to the wavelengths of light required to excite the GFP generated from the *MLC-GFP* plasmid.

filter set. *MLC-GFP* transfected into Cos 7 cells produced no GF (data not shown). The background auto-fluorescence from untransfected cells is shown in Fig. 2B. A bright field image (10× lens) depicts the growing Cos 7 cells (Fig. 2C). In Fig. 2D, Cos 7 cells were transiently transfected with the *CMV-lacZ* vector and the βGal

activity visualized after fixation (Ausubel et al., 1993). Although βGal can be visualized in the Cos 7 fibroblasts, these cells were fixed and treated with exogenous substrate, whereas endogenous GFP (Fig. 1A) was observed with the fluorescence microscope in living cells.

It has recently been reported that a Ser→Thr (S65T) mutation in the GFP chromophore improves GF in bacteria (Heim et al., 1995). We sought to determine if this mutation would fluoresce more brightly than wt GFP in Cos 7 cells. We generated an S65T mutation via PCR mutagenesis which was sequenced then subcloned into the pcDNA3 vector. Nominal differences in GF were observed between the S65T mutation (Fig. 2E) and the wt *CMV-GFP* construct (Fig. 2F) when transiently transfected into Cos 7 cells. Although we did not directly quantitate the fluorescence, we estimate the amount of GF resulting from the S65T mutation to be no more than 2-fold that of the *CMV-GFP* generated GF.

A parallel set of transfections was performed in C2C12 myoblasts (Fig. 3). The *gfp* expression from the *MLC-GFP* construct was evident only after 48 h in differentiation media (Fig. 3A). During this time, transfected C2C12 mono-nucleated myoblasts fused to form myotubes containing several nuclei within a single cytoplasm. GFP was produced throughout the entire cytoplasm in about 10% of the myotubes and was best visualized with a 25× water immersion lens. The reduced resolution of GFP in myotubes when compared with the Cos 7 cells may be due to the larger amount of cytoplasm in the myotubes, to the relative number of *gfp* transcripts produced by the two constructs, or to different translational or post-translational environments in the two cell types. In duplicate transient transfections of C2C12 cells, variability in the GF of different myotubes was also evident (data not shown). The background fluorescence of an untransfected culture of C2C12 myotubes can be seen in Fig. 3B along with a bright field image in Fig. 3C. The *MLC-lacZ* construct was separately transfected into C2C12 myoblasts which were differentiated into myotubes and the βGal activity visualized after fixation (Fig. 3D). When the *CMV-GFP* vector was transfected into C2C12 myoblasts, expression was apparent in dividing cells at about 12 h post-transfection. However, once the cells were differentiated into myotubes in low serum, GF was no longer apparent (data not shown).

(b) Stable C2C12 myotube cultures produce GFP

We determined whether *gfp* would continue to be expressed in mammalian cells when stably integrated into the chromosomes of muscle cells. C2C12 myoblasts were co-transfected in a ratio of 15:1 with the *MLC-GFP* construct and a Hy^R vector, respectively. Six resistant lines were expanded at low density as myoblasts. A portion of each clone was differentiated into myotubes and evaluated for GF. As shown in Fig. 3E and F, myotubes from two differentiated C2C12 stable cell lines displayed variable levels of *gfp* expression. Since we have not evaluated other stably transfected cell types, it is not clear whether the variability in GFP produced was due to myotube formation or is an inherent quality of the protein or its expression. The gene appeared to be stably transmitted and expressed since the cells still fluoresced after three passages in selection media (data not shown).

(c) Transiently transgenic zebrafish produce GFP

We employed the chordate fish *Danio rerio* (zebrafish) as a facile test for expression of the *MLC-GFP* construct in living animals. Because these fish are initially transparent, GF could be assayed directly using the same conditions as for the tissue culture cells. Non-linearized *MLC-GFP* plasmid was injected into fertilized zebrafish eggs and muscle-restricted GF was first observed in mosaic animals after 24 h of development. At this time, the embryos have just straightened and are beginning to flex their tail muscles. The injected embryo in Fig. 4 has developed approx. 30 somites. Its appearance under bright field microscopy can be viewed in Fig. 4. Of more than 50 expressing survivors, all had differing numbers of labeled somitic muscle fibers. The uninjected 36-h embryo in Fig. 4 produced background fluorescence primarily in the yolk cells. GF derived from the *MLC-GFP* construct was not observed in any other tissue of these mosaic animals. This observation is in contrast to embryos injected with the *CMV-GFP* construct where groups of unidentified, non-muscle cells at the ventral border of the yolk sac were labeled with GFP (data not shown).

One of the *MLC-GFP* injected survivors was observed with GF and bright field optics as a fry after 1.5 months of development (Fig. 5). Despite the development of scales, this older zebrafish continued to be transparent to the blue excitation wavelengths necessary to stimulate the visible muscle-specific GF observed in this transient transgenic animal. The *gfp* gene continued to be expressed and/or the protein was stabilized only in skeletal muscle cells.

(d) Conclusions

(*1*) In this study we have demonstrated the use of GFP as a vital dye in muscle cell cultures and transient transgenic zebrafish muscle. The primary advantage of GFP over other available reporters such as *lacZ* is that gene expression can be continuously observed in living cells, while fixed cells are lost to further analysis. Although it is possible to visualize βGal activity in living cells using the substrate fluorescein-di-β-D-galactopyranoside (Molecular Probes), *gfp*-expressing cells can be

observed directly without perturbing the cultures or animals with additional components. We envision that stable cell lines could be rapidly produced using GFP, since GF cells could be identified without drug selection.

(2) Since the GF generated in myotubes by the *MLC-GFP* construct was visible only at a 25-fold magnification or more, this suggested that the protein concentration was lower in these cells than in the *CMV-GFP* transfected Cos 7 cells where GF was visible using a 10× lens. This observation may be due to a combination of a weaker promoter and/or a larger cytoplasm when compared to the Cos 7 transfections. In addition, the expression or post-translational modification of GFP protein, as well as the redox environment could be more favorable in Cos 7 cells. Since muscle cells fuse to form syncytia of many nuclei contained within a single cytoplasm, a relatively strong muscle-specific promoter/enhancer construct such as *MLC-GFP* was capable of producing detectable levels of GFP protein in transient transfections. A weaker 1565-bp myogenin promoter (Cheng et al., 1993) regulating *gfp* expression generated very low levels of GF in transiently transfected C2C12 cells. However, when the *myogenin-GFP* plasmid was injected into zebrafish embryos, muscle-specific GF comparable to that generated by the *MLC-GFP* construct was detected after 24 h (data not shown). Perhaps the construct was integrated into the genome of the embryo such that multiple copies of *myogenin-gfp* were generated during the rapid replication of DNA before the midblastula transition when zygotic genes are transcribed (Westerfield, 1993). This would allow for the production of larger amounts of GFP protein than in transient transfections.

(3) The GFP protein is not toxic to mammalian cells since C2C12 cell lines expressing *gfp* can be passaged several times. Similarly, zebrafish embryos injected with the *MLC-GFP* construct transiently expressed high levels of tissue-specific protein without any deleterious effect. This property of GFP makes it a useful vital marker for the preparation of transgenic animals. Since the introduction of transgenes is very inefficient in zebrafish (Lin et al., 1994), a readily detectable marker would be invaluable. In addition, constructs carrying different promoters regulating *gfp* expression in a cell or tissue-specific manner could be used to follow developmental processes in vivo. Cell migration and morphogenesis could be directly compared in wt and mutant animals.

(4) The *MLC-GFP* stable cell lines generated GF only in myotubes, providing a useful marker for the study of muscle differentiation in cell culture. The timing of other muscle-specific regulatory regions expressing *gfp* could also be assayed in this system. We could not select *gfp*-expressing muscle cells directly since the *MLC* control regions initiate expression only during differentiation, when cells enter G_0 and no longer replicate. Although other constructs such as *CMV-GFP* may be expressed in a number of different zebrafish tissues, the *MLC-GFP* construct can be used in studies of muscle differentiation, since its expression is strictly muscle-specific. One disadvantage of the rat *MLC-GFP* construct is that it is not expressed until relatively late during development in the transiently transgenic zebrafish. Additional zebrafish-specific control elements may be required for accurate timing of expression. Notably, the rat MLC transcriptional control regions present in our construct were capable of directing muscle-specific expression of *gfp* in the zebrafish. This functional conservation of promoter and enhancer activity probably extends to other genes as well. We are currently investigating the use of other GFP muscle markers in the study of muscle differentiation in the developing zebrafish embryo.

ACKNOWLEDGEMENTS

We are grateful for helpful discussions with Mark Fishman. This work was supported by grants to N.R. from the NIH (RO1AR41926 and RO1AG12316) and a sponsored research agreement from Bristol-Myers Squibb. N.R. is an Established Investigator of the American Heart Association. Funding for J.B.M. is through the Muscular Dystrophy Association and NIH postdoctoral fellowship F32HD07926. E.R. is supported by a Human Frontier Science Program postdoctoral fellowship. Part of the work was supported by NSF IBN-9317469 to W.D.

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Cheng, T.-C., Wallace, M C., Merlie, J.P. and Olson, E.N.: Separable regulatory elements governing myogenin transcription in mouse embryogenesis. Science 261 (1993) 215–218.

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Exhibit 3

DEVELOPMENTAL BIOLOGY 192, 289–299 (1997)
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High-Frequency Generation of Transgenic Zebrafish Which Reliably Express GFP in Whole Muscles or the Whole Body by Using Promoters of Zebrafish Origin

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Despite a number of reports on transgenic zebrafish, there have been no reports on transgenic zebrafish in which the gene is under the control of a promoter of zebrafish origin. Neither have there been reports on transgenic zebrafish in which the gene is under the control of a tissue-specific promoter/enhancer. To investigate whether it is possible to generate transgenic zebrafish which reliably express a reporter gene in specific tissues, we have isolated a zebrafish muscle-specific *actin* (*α-actin*) promoter and generated transgenic zebrafish in which the green fluorescent protein (GFP) reporter gene was driven by this promoter. In total, 41 GFP-expressing transgenic lines were generated with a frequency of as high as 21% (41 of 194), and GFP was specifically expressed throughout muscle cells in virtually all of the lines (40 of 41). Nonexpressing transgenic lines were rare. This demonstrates that a tissue-specific promoter can reliably drive reporter gene expression in transgenic zebrafish in a manner identical to the control of the endogeneous expression of the gene. Levels of GFP expression varied greatly from line to line; i.e., fluorescence was very weak in some lines, while it was extremely high in others. We also isolated a zebrafish cytoskeletal *β-actin* promoter and generated transgenic zebrafish using a β-actin–GFP construct. In all of the four lines generated, GFP was expressed throughout the body like the *β-actin* gene, demonstrating that consistent expression could also be achieved in this case. In the present study, we also examined the effects of factors which potentially affect the transgenic frequency or expression levels. The following results were obtained: (i) expression levels of GFP in the injected embryo were not strongly correlated to transgenic frequency; (ii) the effect of the NLS peptide (SV40 T antigen nuclear localization sequence), which has been suggested to facilitate the transfer of a transgene into embryonic nuclei, remained to be elusive; (iii) a plasmid vector sequence placed upstream of the construct might reduce the expression levels of the reporter gene. © 1997 Academic Press

INTRODUCTION

Zebrafish is an excellent model organism for the study of vertebrate development (Kimmel, 1989; Nüsslein-Volhard, 1994). The embryos develop outside the mother and are optically transparent, allowing direct observation of their embryonic development. A relatively short generation time of 2–3 months makes classical genetics feasible. Recent large-scale mutagenesis screening has led to the isolation of a great number of lethal mutations of genes essential for embryonic development (Haffter *et al.*, 1996; Driever *et al.*, 1996).

An important technology in model organisms is the capability of producing transgenic organisms as has been shown for *Drosophila* or mice. Transgenic zebrafish, using several DNA constructs, have been generated in the past 9 years.

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Stuart *et al.* (1988, 1990) first showed that DNA injected into the cytoplasm of fertilized zebrafish eggs could integrate into the fish genome and be inherited in the germline. Culp *et al.* (1991) demonstrated that the frequency of germline transmission of a microinjected transgene could be as high as 20%. Despite these initial successes, however, transgenic zebrafish had the problem of inconsistent expression of a transgene, i.e., variegated expression (Stuart *et al.*, 1990) or no expression (Stuart *et al.*, 1988; Culp *et al.*, 1991). In these experiments, promoter/enhancer sequences of SV40 or RSV (Rous sarcoma virus) origin were used. Lin *et al.* (1994b) used a *Xenopus* elongation factor 1α enhancer/promoter to drive LacZ expression. The expression patterns of LacZ in the four lines obtained were highly variable. Bayer and Campos-Ortega (1992) tried to apply an enhancer trap in zebrafish, using a mouse heat-shock promoter. Although one transgenic fish in which LacZ was expressed in primary sensory neurons was generated, LacZ expression was not fully penetrant. To date, the study by Amsterdam *et al.* (1995) appears to be the most successful in terms of the consistent expression of a reporter gene in zebrafish. They used a modified *Xenopus* elongation factor 1α enhancer/promoter to drive the expression of the green fluorescent protein (GFP) reporter gene (Chalfie *et al.*, 1994), and showed that five of five transgenic lines expressed GFP, apparently in whole bodies, although one line with the highest GFP expression still showed variegated GFP expression.

Recently, Hopkins and her colleagues developed a new method for generating transgenic zebrafish. They showed that murine leukemia virus/vesicular stomatitis virus pseudotyped retroviral vectors can integrate into the zebrafish genome at high frequency (Lin *et al.*, 1994a; Gaiano *et al.*, 1996a). The method has been proven to be useful for insertional mutagenesis (Gaiano *et al.*, 1996b; Allende *et al.*, 1996). However, since retroviral vectors have limitations of vector size, introduction into the genome of a relatively long construct, such as long *cis*-regulatory sequences, would be impossible using this method.

A possible cause for the frequently observed silent or variegated expression of the constructs introduced could, in part, be the use of sequences of heterologous origin. There is an example in which a promoter of zebrafish origin was used to drive the gene expression (Meng *et al.*, 1997). But it was a transient expression analysis; i.e., gene expression was monitored in an injected embryo, not in a germline-transmitted embryo (Meng *et al.*, 1997). To date, no transgenic zebrafish in which the gene is driven by a promoter of zebrafish origin, nor transgenic zebrafish in which a reporter gene is driven by a tissue specific promoter/enhancer, have been generated. Thus, whether the reproducible generation of transgenic zebrafish with tissue-specific transgene expression is possible has not yet been clarified. Given this background, we attempted to introduce GFP driven by a zebrafish muscle-specific actin promoter (*α-actin* promoter) into zebrafish to investigate whether reliable and tissue-specific expression in transgenic zebrafish can be achieved by using a tissue-specific promoter/enhancer of zebrafish origin. Here we show that it is possible to reliably obtain GFP-expressing transgenic zebrafish with the α-actin–GFP constructs. We also show that, by using zebrafish cytoskeletal *β-actin* promoter, it is possible to reliably obtain transgenic zebrafish which express GFP throughout the body.

In this study, we also investigated the effects of the following three factors which potentially affect transgenic frequency or expression levels. The first is whether levels of transient expression in an injected embryo show any correlation to transgenic frequency. The second is whether coinjection of the SV40 T antigen nuclear localization sequence, which has been suggested to facilitate the transfer of a transgene into embryonic nuclei (Collas *et al.*, 1996), has any effect on transgenic frequency. The third is whether the presence of a plasmid vector sequence has any effect on the expression levels of GFP.

MATERIALS AND METHODS

Cloning of Actin Genes from Zebrafish

RACE–PCR was carried out against first-strand cDNA of 14- to 24-h-old embryos using two degenerated primers based on the amino acid sequences, WHHTFY and WDDMEK, which are present in all types of actin proteins. The nucleotide sequences of the primers were TA(A/G)AA(A/G/C/T)GT(A/G)TG(A/G)TGCCA and GGAAGCTT(C/T)TCCAT(A/G)TC(A/G)TCCCA. Nested PCR was performed and four types of DNA fragments, about 300 bp each, were obtained.

An *α-actin* genomic fragment of about 3.7 kb, which includes the first and the second introns, was recovered by PCR using *α-actin*-specific primers. Southern blotting using this fragment as a probe revealed a single 12-kb band. A zebrafish genomic sublibrary was constructed using 10- to 15-kb *Eco*RI genomic fragments and screened with the 3.7-kb probe. Three identical positive clones, 12 kb in length, were isolated.

A *β-actin* genomic fragment of 1.9 kb, which includes the first and the second introns, was recovered by PCR using *β-actin*-specific primers. This fragment was used for the screening of zebrafish genomic libraries which were provided by Drs. Petkovich, Picker, Takeda, and Kikuchi. Several overlapping phage clones were obtained.

Construction of α-Actin–GFP and β-Actin–GFP Plasmids

Two modified GFP sequences, GFP-S65A (Moriyoshi *et al.*, 1996) and EGFP (Clontech), were used. An *α-actin* upstream fragment (*α-actin* promoter; αp) of about 3.9 kb was PCR-amplified from the *α-actin* λ phage clone using the upstream primer, T3, and the downstream primer, TTGGTCTGTGCAGGACAA. The junction between the first intron and the second exon exists within the downstream primer. The initiation methionine codon is located several nucleotides downstream of the downstream primer sequence. Two αp-GFP plasmids, αp-GFP(S65A) and αp-EGFP, were constructed. For the S65A construct, GFP(S65A) followed by an SV40 poly(A) signal was fused to αp. For the EGFP construct, EGFP followed by a BGH poly(A) signal was fused to αp. The backbone plasmid vec-

tors were pBluescript (SK) in both cases. Structural details of the two plasmids are available upon request.

A *β-actin* upstream fragment (*β-actin* promoter; *β*p) of about 17 kb was PCR-amplified from one of the *β-actin* phage clones using the upstream primer, T3, and the downstream primer, AAGGATCCACTGTAAAAGAAAGGGAA. The junction between the first intron and the second exon exists within the downstream primer. The initiation methionine codon is located several nucleotides downstream of the downstream primer sequence. For the poly(A) signal, a *β-actin* 3′ fragment (*β3′*) of about 8.4 kb was used. The upstream end of *β3′* is a *Bam*HI site, which is located within the second exon. The downstream end is an end of one of the phage clones. The exact location of the poly(A) additional signal is unknown. The *β*p-EGFP-*β3′* plasmid was constructed using the pBluescript (SK) backbone plasmid vector. Structural details of the plasmid is available upon request.

DNA Preparation and Microinjection

Plasmid DNA was prepared using the Qiagen plasmid kit (Qiagen). For BS-*α*p-G, the *α*p-GFP(S65A) plasmid was linearized by *Sal*I. For *α*p-G-BS, the *α*p-EGFP plasmid was linearized by *Sal*I. For *β*p-G-*β3′*-BS, the *β*p-EGFP-*β3′* plasmid was linearized by *Xho*I. Linearized plasmid DNA was extracted using phenol–chloroform and then chloroform, precipitated by ethanol, and dissolved in distilled water. For *α*p-G, the *α*p-GFP(S65A) plasmid was digested with *Sac*I and electrophoresed on an agarose gel. DNA fragments of *α*p-GFP(S65A)-pA were recovered from the gel using the GeneClean II kit (Bio 101) and dissolved in distilled water.

Maintenance of wild-type fish and collection of embryos were carried out as described by Westerfield (1993). Microinjection of DNA was carried out using an agarose gel with depressions as a holding plate (Westerfield, 1993). DNA solution of about 25 ng/μl in distilled water was air-pressure-injected into the cytoplasm of a one-cell-stage zebrafish embryo with its chorion intact. The SV40 T antigen nuclear localization sequence (NLS; CGGPKKKRKVG-NH_2) was added to the injection solution to a final concentration of 0.5 ng/μl in more than half of the experiments. The peptide was synthesized and HPLC-purified by Takara Co. In contrast to most previous studies, color dye, such as phenol red or fluorescein dextran, was not added to the injection solution. The injection volume was estimated from the difference in refractive indices between the injection solution and cytoplasm. The injection volume was adjusted such that one-third to half of the injected embryos died or became malformed by the next day; exact volume was unknown. Injections were usually carried out within 20 min after fertilization, when cytoplasm of embryos was not yet high. Fertilized eggs were collected several times (typically 3 times) for one injection session. We injected up to about 70 eggs per egg collection. Thus, when fish continued to produce eggs for 1 h, we were able to inject up to about 210 embryos. When fish produced all their eggs in a limited period, fewer embryos were injected. Usually, 100–150 embryos were injected in one injection session. The next day, live embryos exhibiting very weak GFP expression (such as fish-X in Fig. 3), about one-fourth, were discarded. About one-third of the fish selected for raising died before sexual maturity (usually within a few weeks). Usually 25–40 fish reached sexual maturity per typical injection session.

DNA Isolation and PCR

DNA isolation from pools of 50–200 3-day-old fish or from individual embryos was carried out according to Amsterdam *et al.* (1995). PCR reaction was carried out using two primers within GFP in either case of GFP(S65A) or EGFP, which resulted in the amplification of the 300-bp band from the respective plasmid. All reactions contained primers in the *islet2* gene (Tokumoto *et al.*, 1995) as an internal control. DNA isolation from adult fish for Southern blotting was carried out according to Westerfield (1993).

Fluorescence Microscopy

Embryos in a 9-cm plastic dish were observed using an Olympus IX70-FLA inverted fluorescence microscope with an FITC filter. Usually, the 4× objective lens was used. In some cases, photographs were obtained using an Olympus BX50-FLA noninverted fluorescence microscope.

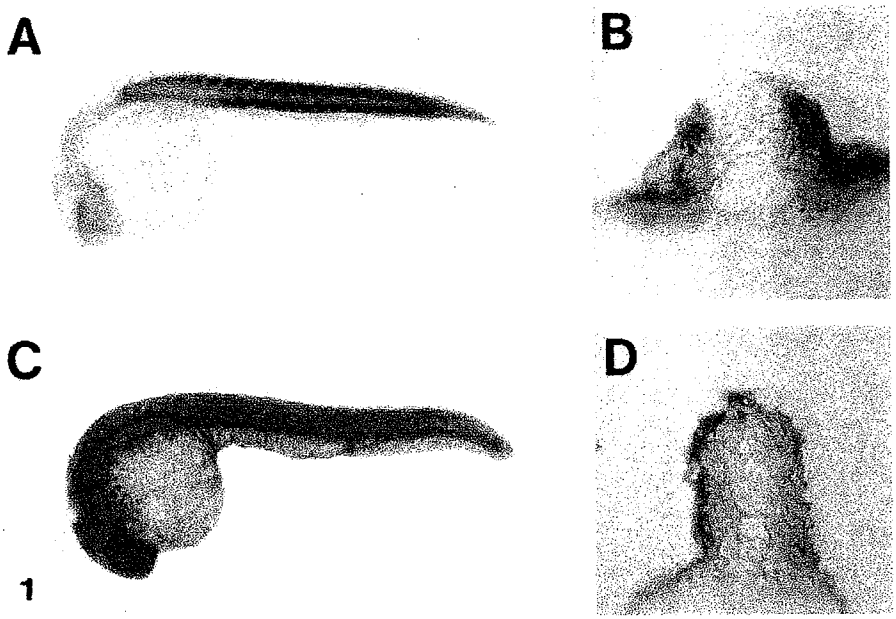
RESULTS

Cloning of Actin Genes from Zebrafish

Four types of *actin* genes were identified by RACE-PCR (see Materials and Methods). They were called as *α1-*, *α2-*, *β-*, and *γ-actin* based on their expression patterns and the sequence similarity to *actin* genes from other species. *α1-actin* is also referred to simply as *α-actin*. The expression of *α(α1)-* and *α2-actin* was muscle-specific, while that of *β-* and *γ-actin* was ubiquitous. Figure 1 shows the expression patterns of *α-actin* (A and B) and *β-actin* (C and D).

Transient Expression of GFP in α-Actin–GFP-Injected Embryos

Genomic sequences flanking *α-actin* were isolated (see Materials and Methods). The structure of the 5′-region of *α-actin* is shown in Fig. 2A. The initiation methionine codon is located near the 5′ end of the second exon (20 bp downstream of the exon–intron junction). A DNA fragment of about 3.9 kb, which contains about 2.2 kb of the upstream fragment, about 40 bp of the first exon, about 1.7 kb of the first intron, and a part of the second exon (5 bp), was used as an *α-actin* promoter (*α*p). The first intron was included for two reasons: one is because intervening sequences have been suggested to increase gene expression in transgenic mice (Brinster *et al.*, 1988) and zebrafish (Amsterdam *et al.*, 1995) and the other is because there may be segments of *cis*-regulatory elements in the first intron. Modified GFP (S65A; Moriyoshi *et al.*, 1996), followed by the SV40 poly(A) signal, was fused to the *α*p. The *α*p-GFP(S65A) plasmid was linearized and injected into one-cell-stage embryos, which were later subjected to fluorescence analysis. Cytoplasmic injection of DNA constructs is known to result in mosaic expression of a reporter gene in zebrafish (e.g., Amsterdam *et al.*, 1995). As expected, fluorescence was observed in a subset of muscle cells in 1-day-old embryos (Fig. 3). Expression patterns were highly variable from embryo to embryo.



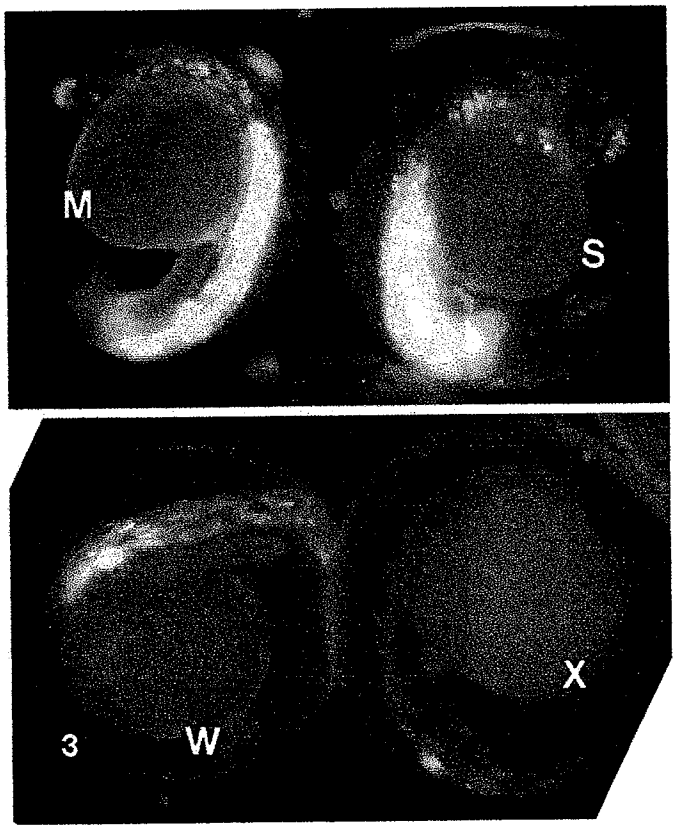


FIG. 1. Expression of *α-actin* and *β-actin* in embryonic zebrafish. One-day-old embryos were subjected to *in situ* hybridization using the *α-actin* (A and B) or *β-actin* (C and D) probe. A and C are whole mounts, while B and D are cross sections.
FIG. 3. Transient expression of GFP in α-actin–GFP-injected embryos. Embryos were viewed through their chorions. S, M, W, and X are the expression levels of GFP in each embryo. S, strong; M, moderate; W, weak; X, very weak, so that the embryo should be discarded.

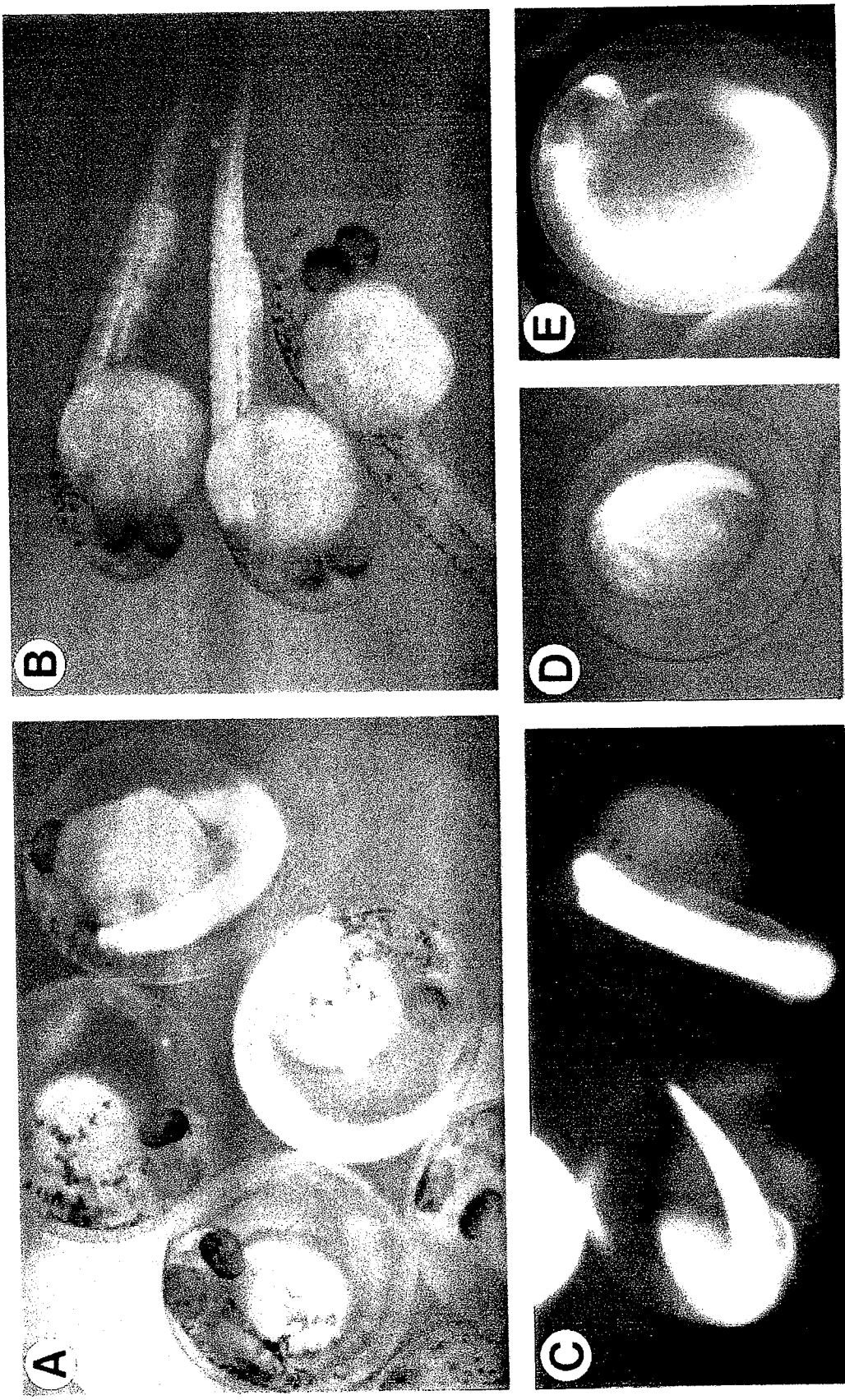


FIG. 4. Expression of GFP in transgenic embryos generated using α-actin–GFP. Embryos were viewed through their chorions, except for (B) where chorions were removed. (A) 30-h-old embryos of a B-rank transgenic line. Nonfluorescent embryos are nontransgenic siblings. (B) 28-h-old embryos of C-rank and D-rank transgenic lines. A nontransgenic embryo is also shown. (C) 26-h-old embryos of an A-rank transgenic line. (D) A 13-h-old embryo of an A-rank transgenic line. (E) A 26-h-old embryo of the most fluorescent line (A-rank). Film-exposure time for this figure was much shorter than that in other figures. Due to the high fluorescence of the muscles, other regions of the embryo are clearly visible.

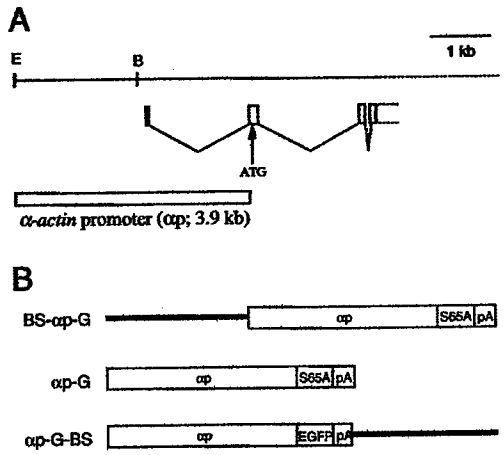


FIG. 2. Maps of *α-actin* and α-actin–GFP constructs used in this study. (A) Structure of *α-actin*. The initiation methionine codon (ATG) is located 20 bp downstream of the intron 1–exon 2 junction. The *α-actin* promoter used in this study (αp) includes the intron 1–exon 2 junction, but does not include the ATG initiation codon. E, *Eco*RI; B, *Bam*HI. (B) Structure of α-actin–GFP constructs used in this study. Thick line, plasmid vector sequence; S65A, GFP(S65A); pA, poly(A) additional signal.

Generation of Germline-Transmitting Fish Using α-Actin–GFP

Three constructs were used to produce stable transgenic zebrafish lines (Fig. 2B). BS-αp-G and αp-G were derived from the same plasmid, αp-GFP(S65A). The difference was the presence or the absence of plasmid vector sequence. The plasmid vector sequence has been suggested to suppress gene expression in transgenic mice (Chada *et al.*, 1985; Townes *et al.*, 1985). We also injected a third construct, αp-G-BS, in which EGFP (Clontech) was used instead of GFP(S65A) and the plasmid vector sequence was downstream of the fragment.

Each construct was injected individually into one-cell-stage embryos, which were raised for 1 day and viewed using an inverted fluorescence microscope. Embryos with few fluorescent cells were discarded. On average, about one-fourth of the injected embryos were discarded. In the case of BS-αp-G or αp-G, the remaining embryos were grouped according to the intensity of fluorescence. The purpose of this grouping is to investigate whether there is any correlation between levels of transient expression and the frequency of appearance of germline-transmitting founders. In more than half of the injections, the SV40 T antigen nuclear localization sequence (NLS; CGGPKKKRKVG-NH_2) was coinjected with DNA. This peptide has been suggested to facilitate the transfer of a transgene into embryonic nuclei (Collas *et al.*, 1996). To investigate the effects of NLS on the frequency of appearance of germline-transmitting founders, injections without NLS were also carried out.

Injected embryos were raised to sexual maturity and screened for germline-transmitting founders. The fish were mated to wild-type fish and the fluorescence of their 1-day-old progeny was examined using an inverted fluorescence microscopy. The frequency of appearance of germline-transmitting founders is summarized in Table 1, showing that germline-transmitting founders whose progeny expressed detectable levels of GFP were isolated in all types of experiments. In total, 41 of 194 (21%) were positive. As has been seen in previous studies of transgenic zebrafish lines (Stuart *et al.*, 1988, 1990; Culp *et al.*, 1991), founder fish had mosaic germlines. Rates of F1 inheritance of GFP-expressing offspring ranged from 2 to 50% (data not shown). For several lines ($n = 5$), DNA from individual offspring was extracted and analyzed for the presence of the GFP sequence by PCR. Every fluorescent embryo proved to be PCR-positive and every nonfluorescent embryo proved to be PCR-negative in all cases (data not shown). We also examined possible transgenic lines which did not express detectable levels of GFP. For this purpose, DNA was prepared from pools of embryos derived from each of those fish which only produced nonfluorescent embryos and analyzed by PCR. In to-

TABLE 1
Generation of Transgenic Zebrafish Using α-Actin–GFP

Construct	NLS	Exp	Frequency of germline transmitting founders with detectable levels of GFP expression		
BS-αp-G	+	S	1/3 (33%)	10/39 (26%)	41/194 (21%)
		M	6/24 (22%)		
		W	3/12 (25%)		
αp-G	+	S	4/26 (15%)	20/105 (19%)	
		M	9/41 (22%)		
		W	7/38 (18%)		
	–	S	3/10 (30%)	6/40 (15%)	
		M	1/9 (11%)		
		W	2/21 (10%)		
αp-G-BS	–	S/M	5/10 (50%)	5/10 (50%)	

Note. Exp, levels of transient GFP expression; S, strong; M, moderate; W, weak.

TABLE 2
Levels of GFP Expression in Transgenic Zebrafish Generated Using Each Construct

Fluorescence level / Construct	Bright A	← B	C →	Dim D
BS-αp-G	1	0	0	9
(total, 10)	(10%)	(0%)	(0%)	(90%)
αp-G	2	13	9	3
(total, 27*)	(7%)	(48%)	(33%)	(11%)
αp-G-BS	2	4	0	0
(total, 6*)	(33%)	(67%)	(0%)	(0%)

Note. One founder generated using αp-G and one founder generated using αp-G-BS produced progeny exhibiting two clearly different expression levels (A and C, A and B, respectively), which are individually classified and listed. Thus, the total numbers of αp-G and αp-G-BS (asterisks) are greater than those listed in Table 1.

tal, 123 fish were analyzed and 3 lines were PCR-positive. Considering that 41 of 194 fish were expression-positive founders, the results indicate that most, if not all, of the transgenic lines expressed detectable levels of GFP in the 1-day-old-embryo stage.

Figures 4A–4E show examples of GFP expression in transgenic lines. Except for one line expressing GFP throughout the body (data not shown), the lines (40 of 41) showed identical spatial expression patterns; GFP was expressed specifically in muscle cells. Variegated expression of GFP was not apparent in any of the lines. Thus, the results indicate that the zebrafish *α-actin* promoter can reliably drive the reporter gene expression in an identical manner as the endogeneous *α-actin* gene in transgenic zebrafish. This is the first demonstration of transgenic zebrafish in which the gene is driven by a tissue-specific promoter.

TABLE 3
Inheritance of α-Actin–GFP in Transgenic Zebrafish Lines

Construct	Line	Inheritance of GFP expression in F2
BS-αp-G	BAG-1	26/50 (52%)
αp-G	AG-1	188/374 (50%)
	AG-2	55/135 (41%)
	AG-3	125/240 (52%)
	AG-4	63/113 (56%)
	AG-5	40/72 (56%)
	AG-6	138/259 (53%)
	AG-7	165/306 (54%)
	AG-8	25/50 (50%)
	AG-9	34/68 (50%)
αp-G-BS	AGB-1	54/102 (53%)
	AGB-2	138/294 (47%)

In contrast to the identical spatial expression patterns of GFP, expression levels varied greatly from line to line. While some fluoresced only weakly (Fig. 4B), some fluoresced extremely strongly (Figs. 4C and 4E). The stage at which fluorescence was first detectable depended on the fluorescence intensity of each line. In the bright lines, GFP expression could be recognized at as early as 10 h, and at 13 h, clear fluorescence in the adaxial cells and somites was observed (Fig. 4D).

The fact that 21% (41 of 149) were GFP-expressing founders suggests that some founders might have multiple integrations. Indeed, at least two founders appeared to have two integrations, since they each produced progeny exhibiting two clearly different expression levels (see Note under Table 2). Southern analysis of F1 progeny from one such founder confirmed that two integrations had indeed occurred in the germline of the founder (data not shown).

Effects of Three Factors Which Potentially Affect Transgenic Frequency or Expression Levels

We examined the effects of three factors which may affect the transgenic frequency or expression levels. Results are summarized in Table 1 (transgenic frequency) and Table 2 (expression level). The first factor is the relationship between levels of transient expression and transgenic frequency. In two experiments using BS-αp-G (NLS+) and αp-G (NLS–), the transgenic frequency of the fish whose transient GFP expression had been strong (S) was higher than average, i.e., 33% (1/3) vs 26% (10/39) and 30% (3/10) vs 15% (6/40), respectively. However, in one experiment using αp-G (NLS+), it was lower than average, i.e., 15% (4/26) vs 19% (20/105). Importantly, transgenic frequency in W (weak) class fish was not very low compared with the average, i.e., 25% (3/12) vs 26% (10/39), 18% (7/38) vs 19% (20/

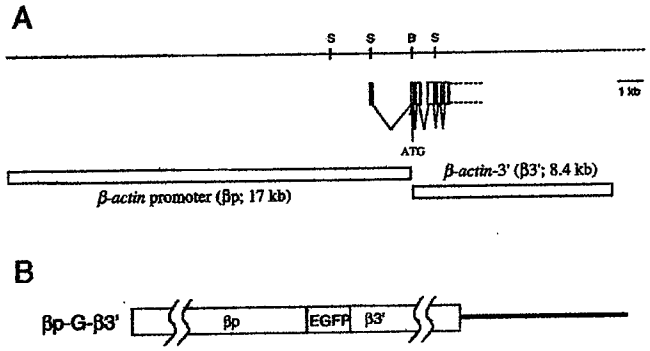


FIG. 5. Maps of *β-actin* and a β-actin–GFP construct used in this study. (A) Structure of *β-actin*. The initiation methionine codon (ATG) is located 8 bp downstream of the intron 1–exon 2 junction. The *β-actin* promoter used in this study (βp) includes the intron 1–exon 2 junction, but does not include the ATG initiation codon. S, *SalI*; B, *BamHI*. (B) Structure of a β-actin–GFP construct used in this study. Thick line, plasmid vector sequence.

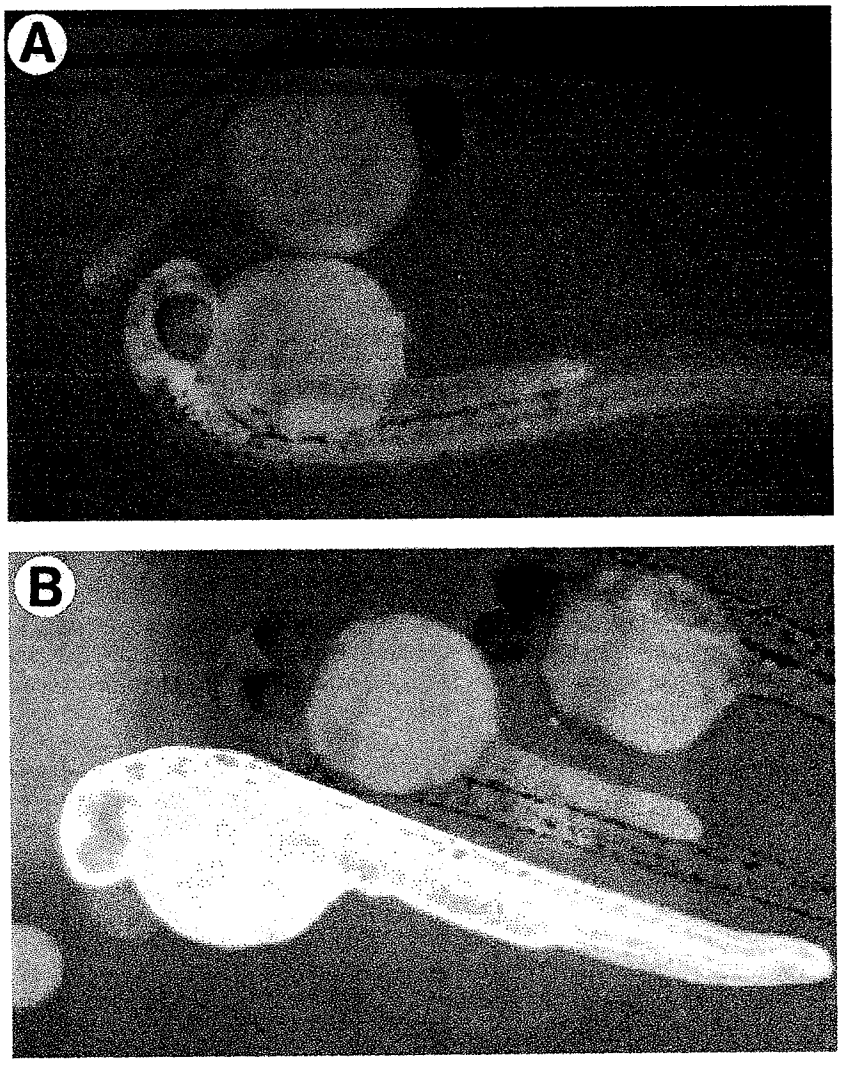


FIG. 6. Expression of GFP in transgenic embryos generated using β-actin–GFP. Chorions were removed. Nonfluorescent embryos are nontransgenic siblings. (A) A typical transgenic embryo at 28 h. (B) A transgenic embryo from the most fluorescent line at 28 h.

105), and 10% (2/21) vs 15% (6/40) in the three experiments. Thus, if there is any correlation, it is not so high that the W class fish should be discarded.

The second one is whether the NLS peptide contributes to an increase in transgenic frequency. NLS+ and NLS− experiments were conducted using the same construct, αp-G. The transgenic frequency in the NLS+ experiment was 19% (20/105), which was somewhat higher than that in the NLS− one, 15% (6/40). However, the difference was not so great that the result was concluded to be positive. Further study is required to determine whether the NLS peptide contributes to transgenic frequency.

The third one is whether the plasmid vector sequence affects the reporter gene expression. Each line was classified into ranks from A–D according to the fluorescence intensity of F1 progeny (Table 2). For the BS-αp-G construct, 9 of 10 lines were classified into the dim D rank, while in the case of the αp-G construct, the D rank was rather rare (3 of 27) and most of the lines were classified into B or C ranks (22 of 27). Considering that BS-αp-G and αp-G were derived from the same plasmid, the results suggest that the plasmid vector sequence has an adverse effect on expression. For the αp-G-BS construct, however, all of the lines (6 of 6) were classified into A or B ranks, despite the presence of the plasmid vector sequence. The high fluorescence was not due to the difference in the GFP sequences used (S65A and EGFP), since mRNA expression levels were also high in the 6 lines generated with the αp-G-BS construct. Thus, the result suggests that the plasmid vector sequence located downstream of the construct does not have an adverse effect on expression.

Maintenance of GFP Expression over a Generation

It is important to determine whether GFP expression is stable after passage through a germline. Thus, fluorescent progeny (F1) of each founder were raised to sexual maturity and mated with wild-type fish. All the lines tested (n = 24) produced fluorescent embryos. Moreover, levels of GFP expression were also completely inherited, i.e., the fluorescence intensity of the embryos (F2 progeny) was the same as that of the parents (F1) when they had been tested for fluorescence. As in previous studies, inheritance of fluorescence in the F2 generation was consistent with the ratio of Mendelian segregation in all the 12 lines where the number of fluorescent and nonfluorescent embryos were counted (Table 3). In three lines, stable transmission of GFP expression has been confirmed in the F3 generation. Taken together, the results strongly support the idea that the transgene is stably integrated into the genome in each line.

F1 progeny of B–D ranks were all healthy. In the case of A rank, a significant fraction of embryos died within 2 weeks. For the reason of the weakness, long exposure of excitation light to A-ranked embryos on a fluorescence microscope could be harmful to embryonic health. However, this is not the only cause of the weakness because those embryos which had been placed in a dark without excitation were still weak. Whether high-level expression GFP itself has toxic effects remains to be determined. If fish could survive in a critical period (a few weeks), most of them were able to become adults and to produce progeny. However, we have not yet succeeded in raising F1 progeny of two lines with extreme GFP expression (Fig. 4E).

Generation of Germline-Transmitting Fish Using β-Actin–GFP

We also generated transgenic zebrafish using the β-actin–GFP construct. A physical map of the zebrafish *β-actin* gene and the construct of the β-actin–GFP transgenic vector are shown in Fig. 5 (for details, see Materials and Methods). We obtained 4 founders out of 53 fish (8%). Three of the four lines exhibited similar GFP expression patterns. GFP was expressed throughout the body in a manner identical to that of the *β-actin* gene (Fig. 6A). In the remaining one line, GFP was also expressed throughout the body, and fluorescence was more intense than that of the other lines (Fig. 6B). In addition, a more prominent expression in the notochord was observed (Fig. 6B). In all of the lines, variegated expression was not observed. Transmission of GFP expression has been confirmed in the F2 generation in two lines.

In summary, we conclude that the *β-actin* genomic sequences used here lead to the reporter gene expression in an identical or near-identical manner to that of the endogeneous *β-actin* gene.

DISCUSSION

Consistent GFP-Expression of Transgenic Zebrafish Generated Using α-actin–GFP or β-actin–GFP Constructs

A number of transgenic zebrafish have been generated in the past 9 years. In the early phase, transgenic zebrafish suffered from either silencing of the transgene expression (Stuart *et al.*, 1988; Culp *et al.*, 1991) or nonconsistent (variegated or highly variable) expression (Stuart *et al.*, 1990; Lin *et al.*, 1994b). More consistent expression was observed by Amsterdam *et al.* (1995), who found that five of five transgenic lines showed near ubiquitous GFP expression in one construct (modified *Xenopus* ef1α enhancer/promoter and the rabbit *β-globin* second intron). In all of the previous studies, promoter/enhancer sequences were not derived from zebrafish origin. There have been no reports of transgenic zebrafish generated using a gene driven by a tissue-specific promoter/enhancer. With this background, we isolated a zebrafish muscle-specific actin promoter and generated transgenic zebrafish using α-actin–GFP constructs. The main aim of this study was to determine whether transgenic zebrafish exhibiting tissue-specific expression can be consistently generated with the use of zebrafish-origin promoters. We have generated as many as 41 GFP-expressing transgenic lines using α-actin–GFP constructs. In virtually all lines established, GFP was expressed in a nearly identical manner to the endogeneous genes; i.e., GFP was specifically expressed in muscle cells. Non-GFP-expressing transgenic lines were extremely rare. These results demonstrate that transgenic zebrafish with consistent expression can be reliably generated using the zebrafish *α-actin* promoter. We also isolated and used the zebrafish *β-actin* promoter. Transgenic zebrafish exhibiting GFP expression throughout the body were reliably generated in this case. This further supports the hypothesis that consistent expression can be achieved by the use of zebrafish-origin promoters.

Establishment of transgenic zebrafish lines using the α-actin–GFP construct is quite easy with the aid of reliable detection of the transgene expression *in vivo*. Screening of one line by merely observing about a hundred embryos through their chorions on an inverted fluorescence microscope usually takes only a few minutes. Transgenic fish can be easily detected among many nontransgenic siblings. Preliminary experiments showed that, at least in transient expression assays, the α-actin–GFP sequence did not affect the expression of another transgene which was driven by several other promoters, when they were placed in the same plasmid. Thus, the addition of α-actin–GFP to one's own constructs could provide a useful *in vivo* marker in the generation of transgenic zebrafish.

In conclusion, we have shown that it is, in principal, possible to consistently generate transgenic zebrafish exhibiting tissue-specific expression of a reporter gene. Since zebrafish has become a popular model organism for the study of vertebrate development, the generation of transgenic zebrafish which express a reporter gene in specific tissues or cells will undoubtedly be useful in the future. In particular, the transparency of the zebrafish embryo will make GFP an excellent marker.

Factors Which Potentially Affect Transgenic Frequency or Expression Levels

We have determined the effects of three factors (levels of transient expression, presence of the NLS peptide, and

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Exhibit 4

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DETERMINATION OF A NECDIN CIS-ACTING ELEMENT REQUIRED FOR NEURON SPECIFIC EXPRESSION BY USING ZEBRA FISH

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To determine cis-acting elements required for neuron specific expression of a necdin gene, we tried to use zebra fish assay system *in vivo* instead of cell lines in vitro. Various expression vectors carrying upstream sequences of necdin gene fused to MEKA (lacZ) gene as a reporter were injected into fertilized zebra fish embryos and then the expression of the reporter gene was analyzed by the whole mount immunochemical method. No promoter activity was obtained with a construct carrying sequence from -63 to +63 of the necdin gene, while promoter activity with preferential skin expression was obtained with a construct having sequence from -86 to +28. Further upstream sequence from -173 to +28 exhibited neuron specific expression as well as that from -845 to +63. These results indicate that a cis-acting element responsible for neuron specific expression is located in an 87bp sequence from -173 to -87 of necdin gene.

A novel necdin cDNA is isolated from P19 embryonal carcinoma cells by the differential hybridization method between untreated and retinoic acid treated cells which are differentiated to neural cells. It encodes 325 amino acids whose molecular size and pI value are about 37 kDa and 8.3, respectively (1, 2). The necdin cDNA as a probe only hybridizes with 1.7kb mRNA of the brain among mouse tissues tested and the differentiated P19 cells, indicating neuron specific expression of necdin (1, 2). *In situ* hybridization experiment also supports the

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neuron specific expression (3). Immunohistochemical analysis reveals that necdin protein is a neuron specific nuclear protein whose expression is seen in postmitotic cells (1, 2). Although the physiological function of necdin protein is unknown, it is interesting to investigate cis-acting elements required for neuron specific expression of necdin, because it is expected to elucidate the mechanism underlying neurogenesis and neuronal differentiation through the transcriptional regulation. Uetsuki et al. (3) have cloned genomic necdin DNA which shows an intronless structure. Transfection of a plasmid carrying an upstream sequence of the necdin gene (from -845 to +63) fused to lacZ gene induce the expression of β-galactosidase in retinoic acid treated P19 cells (3), suggesting that the above upstream sequence contains a region required for neuron specific expression.

Recently, zebrafish, *Brachydanio rerio*, is emerging as a model organism as well as Drosophila and Xenopus laevis. A genetic linkage map for zebrafish and several neuronal mutants have been reported (4, 5). Zebrafish is a useful animal for neuroscience as following reasons ; 1) breeding is easy. 2) fertilized embryos are available every day. 3) primary neurons are formed within 24hr. 4) whole mount analysis is easy, because of the lucid body. 5) it is possible to inject plasmid DNA into more than one hundred of fertilized embryos. By using zebrafish, analysis of GAP-43 promoter activity have been challenged (6). We also attempted to use zebrafish for analyzing cis-acting DNA elements required for neuron specific expression of necdin gene.

MATERIALS AND METHODS

The zebra fish: The Oregon AB line, were maintained at 28.5℃ as described in (7). Expression vector suspended in 25 μg/ml of 2mM Na-phosphate buffer, pH 7.4, 10 mM KCl, 0.01% phenol red and 0.2% ethanol was injected into about 150 fertilized embryos about 500pl in one experiment by Narishige microinjector. Analysis of transgene expression was carried out as described by Reinhard et al (6). Efficiency to produce transient transgenic fish and positive cells numbers per embryo with various constructs were 5.8-18.3% and 1-32 cells, respectively, which

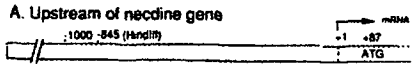
are in a range of reported experiment (6). In this experiment, the above values and transcription level were decreased with progressive deletion of 5' upstream region of the necdin gene.

Preparation of various necdin promoter/enhancer gene plasmids: Necdin promoter, both orientations (Construct 9 and 10 in Fig. 1), extended from -845 to + 63 fused to lacZ gene were constructed at HindIII site in pBluescript SK(-) plasmid (3). Additional constructs containing MEKA cDNA (8) as a reporter gene was prepared as follows; Expression plasmid pcDNAI having CMV promoter (Pcmv) was digested with EcoRI/EcoRV at polylinker site. It was ligated with MEKA cDNA encoding a full length of MEKA protein, and used as a control (Pcmv-MEKA/pcDNAI). The control vector was digested with AflIII, blunt ended and ligated with HindIII linker. It was digested again with HindIII in the presence or absence of BamHI to remove Pcmv and then ligated with HindIII digested necdin promoter (from -845 to +63) prepared from the pBluescript SK(-) or with HindIII/BamHI digested PCR products carrying various length of the necdin promoters. Synthetic oligonucleotide primers having HindIII site or BamHI site for PCR were as follows : 5' GCAAGCT (-291 of necdin gene)TGCCC AAACTCAGTGTGTCCG, 5' GCAAGC (-173)TTTACATAGCCTACTGGTACC, 5' GCAAG (-123) CTTTGACTCTTCGGCTCCTTTC, 5'GCAAGC(-86)TT CTGG CTTCCCCAACACGCA, 5'GCGGAT(+28)CCCTCGGTGGAGACCAGCAG, and 5' GC GGATCCT (+1) GCGCTTTACTGAGCACTGCG.

Whole mount immunochemical procedures : It was performed according to the methods reported (9,10) with a slightly modification. Briefly, to remove the chorion, embryo was treated with 1mg/ml of pronase for 2min at room temperature and fixed with 4% paraformaldehyde containing 0.2% glutaraldehyde, 4% sucrose, 0.15mM $CaCl_2$ and 0.1M Na-phosphate buffer (pH 7.4) for more than 4hr. It was washed with 100 mM Na-phosphate buffer (pH 7.4) containing 0.85% NaCl and 0.3% triton X-100, and then with PBST (20 mM Na-phosphate buffer, pH 7.4, 0.85% NaCl and 0.3% triton X-100) for 60min. To reduce back ground, the sample was further incubated with PBSTS (PBST containing 0.5% skim milk) for 60min. After over night incubation at 4℃ with anti-MEKA, anti-β-galactosidase (both antisera did not react with endogeneous proteins in normal zebrafish embryo) or anti-HNK serum in PBSTS, the sample was washed with PBSTS. Second antibody conjugated with horseradish peroxidase was reacted and then washed with PBSTS, PBST and PBS/0.1% triton X-100. Antigen in embryo was visualized indirectly with 0.1mg/ml of DAB in PBS/0.1% triton X-100 in the presence of 0.05% hydrogen peroxide. The sample was treated with methanol and then benzyl benzoate : benzyl alcohol (2:1), and provided for photograph.

RESULTS AND DISCUSSIONS

Transfection of a plasmid containing specific promoter region into various cell lines and subsequent determination of a reporter gene expression is the well accepted method for the determination of promoter/enhancer region. However, it has been reported that when neurofilament (NF) promoter fused to lacZ gene is transfected into NF-producing PC12h cells or NF-nonproducing C6 cells, C6 cells



B. Constructs of necdin-reporter gene and its expression

		Expression level	Expression in the Brain/spinal cord	Skin	Muscle	Others
1	-845 +63 MEKA	+++++	+++++	-	-	-
2	-291 +28 MEKA	+++	+++++	-	-	-
3	-173 +1 MEKA	-	-	-	-	-
4	-173 +28 MEKA	++	++++	-	-	+
5	-113 +28 MEKA	++	++	++	-	+
6	-86 +28 MEKA	++	+	+++	-	+
7	-63 +63 lacZ	-	-	-	-	-
8	-86 +63 lacZ	++	+	+++	-	+
9	-845 +63 lacZ	+++++	++++	-	+	-
10	+63 -845 lacZ	-	-	-	-	-

Fig. 1. Summary of the expression level and tissue specific expression directed by various constructs of necdin promoter/reporter gene from 3-6 experiments. Upstream of necdin gene with HindIII site and ATG codon (A) and constructs of necdin-reporter gene and its expression in embryos at 24hr (B) are illustrated. Construct 1 was used as a control of expression level (+++++ : as a 100%). The tissue specific expression found in the brain/spinal cord, skin, muscle and others was calculated from total stained cell numbers and illustrated as + (0-20%) to +++++ (80-100%).

express 40 times higher β-galactosidase activity than the PC12h by unknown mechanism (11). In transgenic mice, however, the NF promoter directs neuron specific expression of CAT-reporter gene (12). The results indicate that analysis of promoter region *in vitro* by using cell lines does not always reveal tissue specific gene expression. Therefore, we attempted to utilize zebrafish for determining neuron specific element of necdin gene as an *in vivo* assay system.

At first, various constructs (Construct 1 to 10 in Fig. 1B), carrying different length of the necdin promoter region fused to MEKA or lacZ gene as a reporter, were prepared to identify cis-acting elements responsible for neuron specific expression. The cis-acting elements are expected to exist in the 5' flanking region,

because of the intronless structure of the necdin gene (3). The constructs were injected into fertilized zebrafish embryos and tissue specificity was assayed by counting the cell numbers expressing the reporter protein in different tissues. The results including expression level were summarized in Fig. 1B.

We injected Constructs 1 (MEKA as a reporter) and Construct 9 (lacZ), both of which contain from -845 to +63 of the necdin gene in each expression vector, into fertilized zebrafish embryos and assayed the reporter gene expression in 24hr embryos by the whole mount immunochemical method. Although mosaic expression pattern was observed, more than 80% among the stained cells were restricted to the primary sensory neurons, Rohon-Beard cells and trigeminal ganglion neurons, and neurons in the brain (data not shown). They are dominant neurons which are formed in early developmental stage and their location were confirmed by staining them with anti-HNK serum as a neural cell membrane marker (13). On the other hand, the expression directed by Pcmv-MEKA/pcDNAI as a control vector was observed preferentially in the skin and less than 10% in the primary sensory neurons (data not shown). During the investigation, we noticed that the injection of more than 100 μg/ml concentration of vectors resulted in malformation significantly, and also the linearized or freeze-thawed vectors resulted in the reduction of neuron specific expression with increasing of skin expression (data not shown).

Next, we examined the time-course of the reporter gene expression with Construct 1. Embryos were fixed at 22, 24 and 28hr and then analyzed. Additional neural cells, motoneurons and interneurons were stained in 28hr embryos (Fig. 2), compared to those in 22 or 24 hr embryos which were mentioned above. These results indicate that mouse necdin promoter region spanning from -845 to +63 functioned in zebrafish embryos in temporal, spatial and tissue specific manner. The

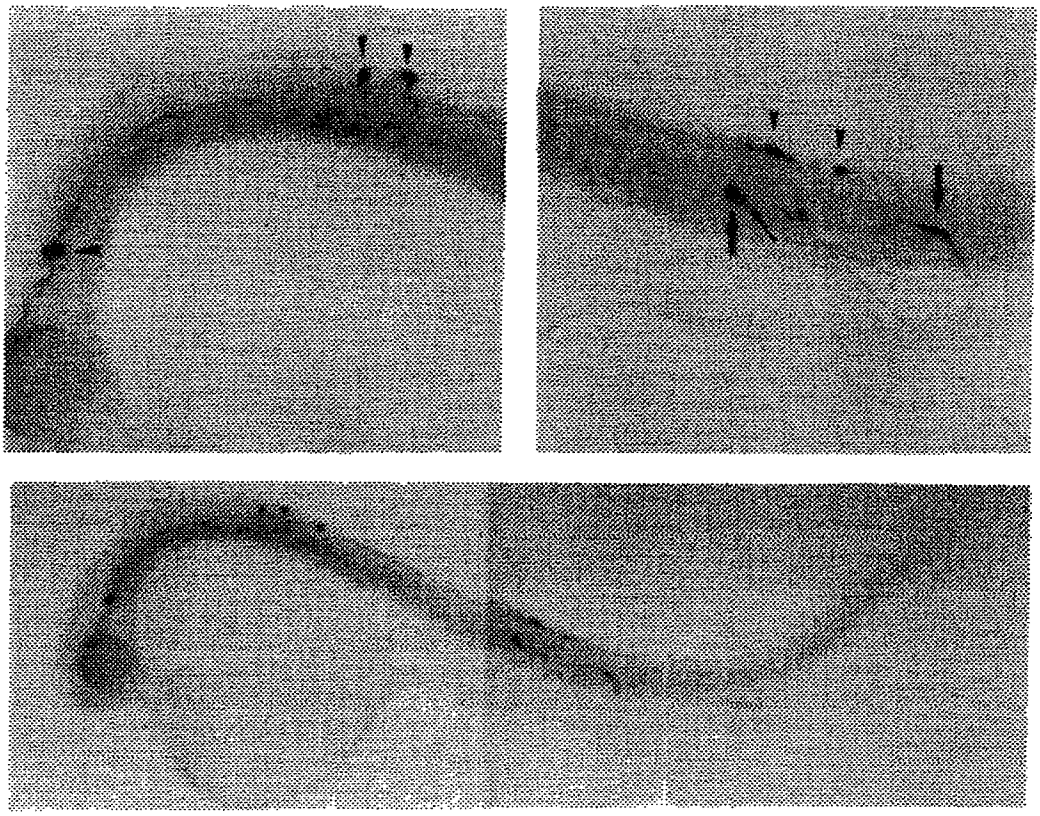


Fig. 2. Expression of MEKA protein directed by necdin promoter in zebra fish. Necdin promoter extended from -845 to +63 fused to MEKA cDNA as a reporter gene was prepared (Construct 1) and MEKA expression was assayed in zebra fish at 28hr. One of the typical embryos reveals 23 positive cells consisted of 22 neurons with neurites and one unidentified cell. Trigeminal ganglion neuron, Rohon-Beard cell, motoneuron and interneuron are indicated by arrowhead, small arrowhead, arrow and small arrow, respectively, in upper enlarged photographs.

promoter activity was dependent on direction of the cis-element, since Construct 10 with a inverted sequence showed no promoter activity.

In the present study, MEKA cDNA is used as a reporter gene. The MEKA protein, identical to phosducin (14), form a complex with $\beta\gamma$ subunits (15,16) of guanine nucleatid-binding proteins, Gt , Gs or Gi. Therefore, the expression of MEKA protein is speculated to inhibit the neurite outgrowth through increasing free Goα concentration in growth cone (17). However, we could not detect any significant morphological changes in zebrafish neural cells and neurites that

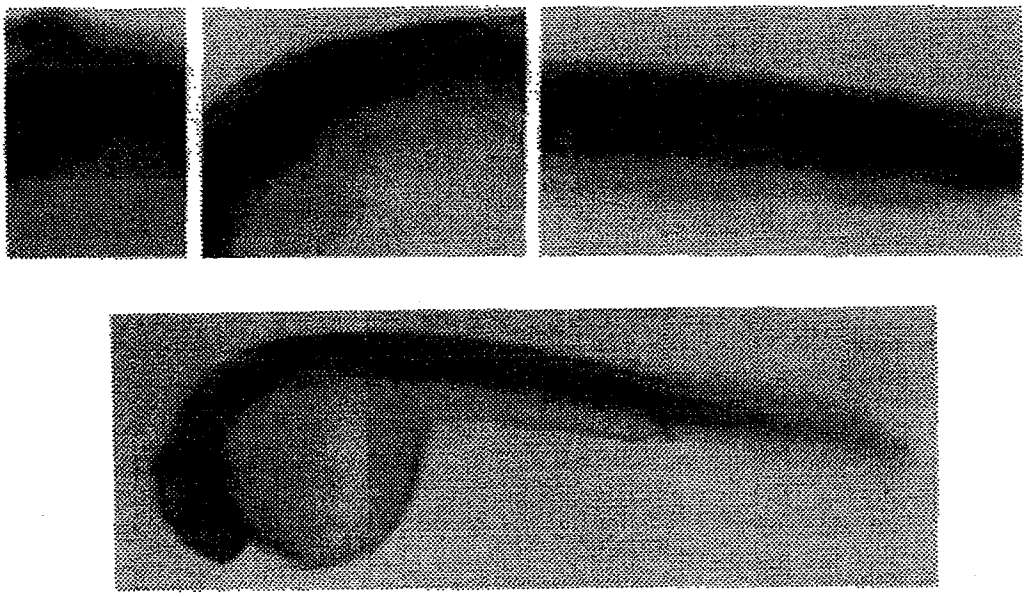


Fig. 3. Neuron specific expression induced by necdin promoter extended from -173 to +28. Expression vector containing necdin promoter (-173 to +28) fused to MEKA cDNA (Construct 4) was injected into fertilized zebra fish embryos and assayed at 24hr. One of the typical embryos reveals 13 positive neurons with neurites.

expressed MEKA protein. One of the possible explanations may be the inability of MEKA protein to associate with $\beta\ \gamma$ subunits of Go.

Expression level of the reporter gene, which reflects the transcriptional activity, was gradually decreased with progressive deletion of 5' flanking region of the necdin promoter (Constructs 1 to 6 in Fig. 1 corresponding to -845 to - 86), and no exppression was observed with Construct 7 (from -63 to +63) in accordance with those of the Uetsuki et al (3). A 5' flanking region from +63 to at least +28, but not to +1, was necessary to retain the promoter activity (compare with Constructs 3 and 4) and may be involved in ribosomal RNA binding.

The shortest fragment required for neuron specific expression among the tested plasmids was observed in Construct 4 (from -173 to +28) as shown in Fig. 1 and

3. In conjunction with the result that Construct 6 (from -86 to +63) showed the promoter activity with preferential skin expression, we concluded that the cis-acting element should locate in an 87bp sequence from -173 to -87 of necdin gene. No homologous consensus sequence was found in the 87bp from known DNA elements including the neuron-restrictive silencer element (NRSE) reported by Mori et al (18). Physiological function of six CTTX repeats found in the 87bp of necdin gene is unknown.

It is interesting to note that Constructs 6 and 8 (from -86 to +63 and from -81 to +63, respectively) almost lost the neuron specific expression in spite of skin expression in this study, although the Construct 8 still causes neuron specific expression in the *in vitro* assay system using retinoic acid treated P19 cells (3). It is suspected that the association of several proteins interacting with multiple DNA elements within the 87bp of the necdin gene is necessary for producing neuron specific expression, because further 50bp deletion (Construct 5 in Fig. 1) from the 5' region of the necdin gene (Construct 4) still exhibited preferential neural expression. Therefore, the different results between *in vitro* and *in vivo* assay systems may be due to the lack of protein(s) which interacts with multiple DNA sites. Difference between Constructs 4 and Constructs 5/6 seems to involve in a switch from neuron specific to preferential skin expression (Fig. 1), indicating the possibility that there is another regulatory element(s) from -173 to -87 for regulating neuron specific expression.

In the present report, we demonstrated by the analysis of necdin gene that zebrafish is a useful screening system to determine a unique enhancer/promoter region, especially an element required for neuron specific expression, because of a rapid development of the primary neurons in a day.

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Exhibit 5

Neurochemical Research, Vol. 21, No. 2, 1996, pp. 231–237

C44

Neuron-Specific Expression of a Chicken Gicerin cDNA in Transient Transgenic Zebrafish*

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Gicerin, a novel cell adhesion molecule which belongs to the immunoglobulin superfamily, is expressed temporally and spatially in the developing chick brain and retina. The previous in vitro experiments using transfected cells showed that gicerin can function as a cell adhesion molecule which has both homophilic and heterophilic binding activities. For the in vivo analyses of gicerin in neural development, we tried to utilize a zebrafish system, a vertebrate suitable for studying early development. We generated transient transgenic animals by microinjecting DNA constructs into zebrafish embryos. Chicken gicerin, under control of the neurofilament gene promoter, was preferentially expressed in neuronal cells and gicerin-expressing neurons exibited a fasciculation formation with neighboring gicerin-positive axons, which may be partly due to homophilic cell adhesion activity of gicerin. These experimental results suggest that this fast and efficient transgenic animal system is useful for studying the functional roles of neuron-specific genes during the development.

KEY WORDS: Gicerin; neural cell adhesion molecule; transgenic zebrafish; functional analysis.

INTRODUCTION

Based on a variety of in vitro experiments and their patterns of expression in vivo, cell adhesion molecules have been implicated as playing major roles during the generation of neural specificity, including neurite outgrowth, growth cone guidance and target recognition (1). However, genetic analysis in both *Drosophila* and mouse has not yet completely evaluated these functions. Some advanced genetic approaches in *Drosophila* are providing a clue to understanding the in vivo roles of neural cell adhesion molecules. For example, ectopic expression of a member of immunoglobulin superfamily showed an altered pattern of growth cone guidance in certain neurons (2). Such approaches, however, are not always practical for the investigation of vertebrate molecules.

Transgenesis has been attempted in many vertebrates by injecting plasmid DNA into fertilized eggs, raising embryos to adults, breeding the offspring, and screening them for transmission of injected sequences. However, the long generation time of some animals makes this approach time-consuming and cumbersome. Furthermore, if the gene product to be expressed is fatal to the organism, it is impossible to establish stable transgenic animals.

Here we describe a convenient method for in vivo analyses of the roles of cell adhesion molecules in the vertebrate neural development, using transient transgenic

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fish, that allows transgene expression in a directed pattern in normally developing nervous system. The zebrafish is well suited for the study of development, especially neuronal development. Hundreds of eggs can be produced daily on a year-round basis from a small number of adult fish. The rapid development of the virtually transparent embryos makes it possible to unambiguously identify many cell types as early as one day after fertilization. Recently, some approaches using transient transgenic zebrafish are providing a possibility of its usefulness in the analyses of mammalian genes, and their organization and expression pattern are highly conserved from fish to mammals (3,4,5). Our aim in the present study was to use this rapid and convenient zebrafish system to test the potential roles of chicken gicerin in neural development by directing its expression in the zebrafish nervous system.

In the previous studies, we have reported on a neurite outgrowth factor (NOF) and its receptor, gicerin, in chicken. Gicerin was first identified as a binding protein for NOF, a member of the laminin family of extracellular matrix proteins (6). Gicerin is an integral membrane glycoprotein of about 82 kD that is expressed temporally and spatially in the developing nervous system (7,8). By isolating and sequencing a gicerin cDNA, we have found that this protein is a novel member of the immunoglobulin superfamily. The in vitro experiments using L-cells transfected with gicerin cDNA showed that gicerin can function as a cell adhesion molecule which has both heterophilic and homophilic binding activities (9,10). These results lead to the suggestion that gicerin might play a role during axonal guidance, since they are expressed on both growth cones and axon pathways they follow.

For the further understanding of the in vivo roles of gicerin, we have generated transient transgenic animals by microinjecting chicken gicerin cDNA constructs into one-cell zebrafish embryos. The murine neurofilament promoter (NFP) was used to target the expression of transgene to the neural tissues. We investigated the expression pattern of gicerin in the developing zebrafish nervous system at cellular levels with whole-mount immunohistochemistry using a specific anti-gicerin antiserum.

EXPERIMENTAL PROCEDURE

Animals. Zebrafish were raised and maintained in aquaria at 28.5 °C on a 14h light/10h dark cycle essentially as described in the Zebrafish Book (11). Fertilized eggs were collected each morning and raised in an embryo-rearing solution (1/3 Ringer's solution, pH 7.2). Developmental stages (h) were expressed as hours after fertilization at 28.5 °C.

Recombinant DNA Constructions. All procedures were done following the standard recombinant DNA techniques (12). An expression vector pcDNA1 (Invitrogen) was digested with *AflIII* and treated with T4 DNA polymerase to produce blunt ends and subsequently *HindIII* linkers were added. The resulting plasmid was inserted by a *XbaI* fragment of the *E. coli* β-galactosidase reporter gene (lacZ) at the *XbaI* site (13). Then the CMV promoter region was removed from this plasmid by digestion with *HindIII* and ligated to make a promoter-proving vector, containing lacZ reporter gene downstream from the *HindIII* site. To construct pNFP-lacZ, a 1.7-kb *HindIII* fragment containing the promoter and enhancer of the murine neurofilament gene (14) was inserted into the *HindIII* site of this plasmid. For the construction of pNFP-gicerin, pNFP-lacZ was digested with *XbaI*, religated, and then inserted by a *BamHI/EcoRI* fragment containing the full-length chicken gicerin cDNA sequence (9) at the comparable site of the plasmid (Fig. 1).

Microinjection and Detection of β-Galactosidase Activity. For injection, recently fertilized zebrafish eggs were pipetted onto an agarose ramp formed by resting glass slides in the lid of 100-mm Petri dish containing molten 1% agarose. Supercoiled plasmid DNA was injected into embryos prior to first cleavage with the aid of a micromanipulator. The injection solution contained 0.5% phenol red (used to estimate the volume of the injection) and 25 μg/ml DNA in 0.1 M KCl (15,16). To determine the patterns of β-galactosidase expression, embryos were fixed at 24 h and assayed for β-galactosidase activity essentially as described by Westerfield et al (3). Briefly, embryos were fixed in 4% paraformaldehyde, 4% sucrose, 0.15 mM $CaCl_2$, and 0.1 M sodium phosphate buffer (pH 7.2) for 1 hr at 4°C. The embryos were then rinsed in 0.1 M sodium phosphate, incubated in 1 mg/ml 5-bromo-4-chloro-indoyl-β-D-galactopyranoside (X-Gal; Stratagene), 150 mM NaCl, 1 mM $MgCl_2$, 1.5 mM $K_4[Fe_2(CN)_6]$, 1.5 mM $K_3[Fe_2(CN)_6]$, in 5 mM sodium phosphate buffer (pH 7.2), at 37°C for 3 hours, fixed again, and then mounted in 50% glycerol and 0.1 M phosphate buffer. Individual transgene-positive cells were counted and assigned to specific tissues, according to their locations and morphologies. Embryos that were obviously malformed were excluded from the analyses.

Whole-Mount Immunohistochemistry. To provide a three-dimensional view of the distribution and morphology of gicerin-positive cells, we reacted whole embryos as following procedures. Embryos were dechorinated by treating with 1 mg/ml pronase for 1 min, rinsed several times, and fixed for 4 hr with 4% paraformaldehyde in 0.1 M phosphate buffer containing 0.15 mM $CaCl_2$ and 4% sucrose (pH 7.2). They were rinsed with 0.1 M phosphate buffer and then with distilled water, and permeabilized with acetone at −20°C for 7 min. After rinsing with distilled water, endogenous peroxidase was inactivated by treating the embryos with 0.3% hydrogen peroxide in methanol for 30 min. The embryos were rinsed with distilled water once more, then with 0.1 M phosphate buffer, and treated with 2% normal goat serum in phosphate-buffered saline (pH 7.2), 1% BSA, 1% DMSO and 0.1% Triton X-100 for 30 min to block non-specific binding sites. Following blocking, the embryo was incubated overnight at 4°C in the primary antibody, rinsed, incubated for 6 hr in biotinylated goat anti-rabbit secondary antibody, and then for 1 hr in horseradish peroxidase (HRP) avidin-biotin complexes (Vectastain Elite ABC kit, Vector Laboratories). The peroxidase reaction was carried out with diaminobenzidine (DAB) as the chromogen. To clear the embryos, they were immersed in a 1:2 mixture of benzyl alcohol and benzyl benzoate after dehydrating with 100% methanol. In the case of double-imunostaining, embryos were serially immunostained with anti-gicerin antiserum, alkaline phosphatase conjugated secondary antibody, and its substrate kit IV (BCIP/NBT,

Fig. 1. Recombinant DNA constructs used to generate transient transgenic zebrafish embryos. To direct expression of transgenes to the nervous system, the 1.7kb of the murine neurofilament gene promoter (NFP) was used. The construct NFP-lacZ contains the NFP (lightly striped boxes) fused to *E. coli* lacZ reporter gene (striped box), whereas the construct NFP-gicerin contains the NFP cloned in the front of the chicken gicerin cDNA (solid box). polyA (open boxes); simian virus 40 polyadenylation site.

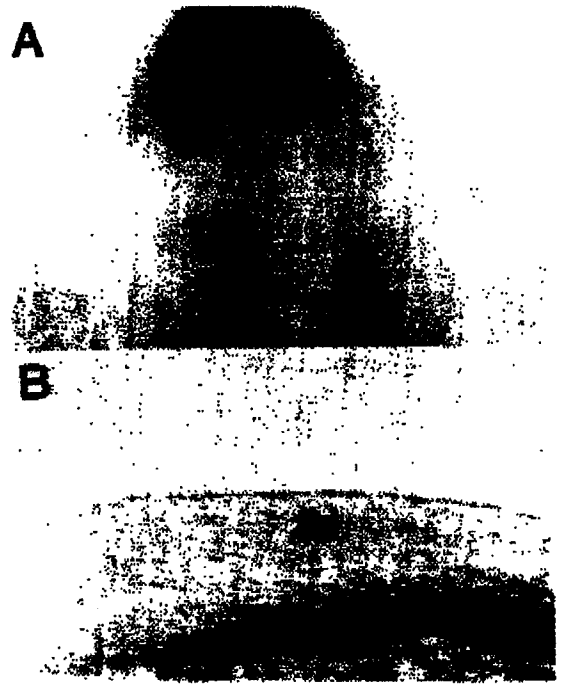

Fig. 2. The murine neurofilament gene promoter (NFP) directed the expression of a marker gene, β-galactosidase, to neuronal cells in transient transgenic zebrafish embryos at 24h. The β-galactosidase activity was detected by X-gal staining of the transgenic embryos in a whole-mount preparation. A, The NFP-lacZ transgene was activated in a trigeminal sensory ganglion neuron located caudal to the developing eyes (asterisks). Horizontal view, rostral is the bottom. B, A transgene-positive Rohon-Beard neuron in the dorsal region of spinal cord in a transgenic embryo. Materials of ventral region are yolk granules. Side view; rostral is to the right and dorsal is up.

Vector Laboratories) which develops into blue color. Then the embryos were double-stained with a monoclonal HNK-1 antibody which could stain almost developing neurons, next with a biotinylated goat anti-mouse IgM secondary antibody, and HRP staining as explained above. Unless otherwise stated, all incubations and rinses were carried out at room temperature on a shaker table. Individual expressing cells were counted and identified according to their locations and morphologies using Nomarski differential interference contrast microscopy.

RESULTS

Activation of Murine Neurofilament Gene Promoter in Zebrafish. Tissue-specific promoters of zebrafish itself are at present not available for the directed expression of transgenes. We first examined whether the activation of murine neurofilament gene promoter (NFP) was restricted to the zebrafish nervous system, as previously described for its expression in mice (14). The putative endogenous fish neurofilament proteins, which can be detected with antisera derived from the mammalian neurofilament proteins, is known to be expressed in the central and peripheral neurons (17, 18). We visualized the activity of NFP in zebrafish by microinjecting recombinant DNA constructs into embryos at the one-cell stage and then assaying β-galactosidase expression in the embryos after fixation at 24 h. Fig. 2 shows transient transgenic embryos in which the expression of lacZ transgene was directed to neuronal cells in the nervous system. The number of cells expressing the transgene was varied from 1 to 20 cells, dependent on individual embryos, probably due to an uneven, mosaic distribution of the injected DNA (3). The expression of NFP-lacZ was prominently detected in trigeminal ganglion neurons (Fig. 2A) and Rohon-Beard neurons (Fig. 2B), both of which are primary sensory neurons that mediate touch sensitivity. These neurons initiate axogenesis at the early time, and appear to be the first neurons in the zebrafish embryo (19). As an expression marker, we used a lacZ gene encoding β-galactosidase, the most commonly used enzymatic reporter molecule.

Since the β-galactosidase was not readily transported into axons in transgene-positive neurons, axons from the β-galactosidase expressing neurons were hardly stained (Fig. 2). We then examined the distribution of all transgene-positive cells which were classified into 5 different type of cells as described in the legend of Fig. 3, and counted 114 cells in 51 animals for NFP-lacZ, and 260 cells in 72 animals for NFP-gicerin. The percentage of transgene-positive cells was plotted against the classified cells in each category. We found that transient transgenic zebrafish embryos preferentially activated NFP in the nervous system. In respect to its expression level, these NFP-drived transgenes showed high levels of expression in neurons compared to that of the other type of cells, if any (data not shown). Our results suggest that the murine neurofilament gene promoter is functionally working in zebrafish and is activated within neurons as seen in transgenic mice. This indicates that transcription factors in zebrafish have an ability to recognize *cis*-acting elements of murine NF gene.

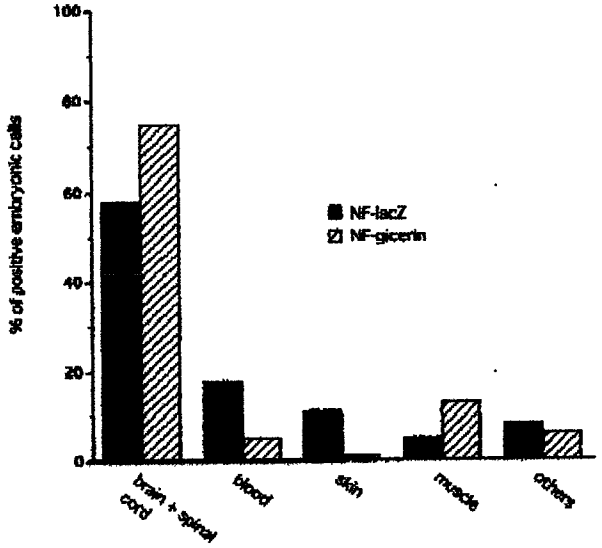

Fig. 3. Tissue distribution of cells expressing transgenes directed by the neurofilament promoter (NFP). The percentage of the total number of transgene-positive cells was plotted against 5 classified cell types where 114 cells in 51 animals were counted for NFP-lacZ and 260 cells in 72 animals for NFP-gicerin. Nervous system cells were identified on the basis of their locations within the brain or spinal cord. Blood cells were identified by their rounded morphologies and locations in the blood stream. Skin cells were the most superficial, flattened cells of the embryo. Muscle cells were identified by their elongated morphologies and striations. Cells that could not be placed into one of these four categories or that were ambiguous to identify were classified as others.

Neuron-Specific Expression of Chicken Gicerin in Zebrafish. We also used an NFP for the directed expression of chicken gicerin in the developing nervous system in zebrafish. Chicken gicerin was expressed exclusively in neuronal cells one day after transgene injection, when large numbers of neurons become postmitotic and extend neurites (19). No gicerin-positive endogenous materials were detected in normal zebrafish embryos when stained with an anti-chicken gicerin antiserum (n = 50, data not shown). As shown in Fig. 4, the expression of chicken gicerin in zebrafish was temporally and spatially regulated by the murine neurofilament gene promoter. The NFP-gicerin transgene was detected in the developing neuronal cells around 20h of embryonal stage when primary neurons differentiated and started to project their axons. On the beginning of differentiation, neuroepithelial cells in the hindbrain of a transgenic zebrafish embryo were stained weakly with an anti-gicerin antiserum (Fig. 4A). Through 22h and 24h of the embryonal development, gicerin-expressing Rohon-Beard neurons projected their central axons both anteriorly and posteriorly, and participated in the formation of the longitudinal axon tract in the spinal cord (Fig. 4B and C). The peripheral axons from a Rohon-Beard neuron formed an extensive arborization on the surface of body, as a general pattern of primary sensory neurons (Fig. 4D).

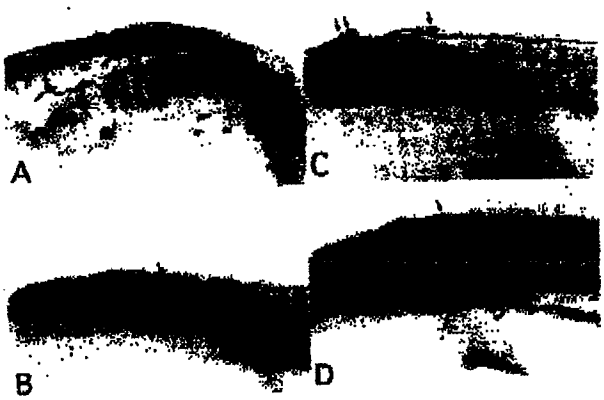

Fig. 4. Time course of gicerin expression under the control of neurofilament gene promoter. The NFP-gicerin transgene was detected in the developing neuronal cells around 20h of embryonal stage (A). Differentiating neuroepithelial cells (asterisks) in the hindbrain of a transgenic zebrafish embryo were stained weakly with the anti-gicerin antiserum. The cell bodies of transgene-positive neurons are indicated by arrows. Through 22h (B) and 24h (C and D), gicerin-expressing Rohon-Beard neurons projected their central axons bidirectionally and participated in the formation of the longitudinal axon tract in the spinal cord (arrowheads), while their peripheral axons (open arrowheads) branched in the skin. Peripheral axons of neurons in (C) are out of the plane of focus. In all panels anterior is to the left, and dorsal is upwards.

Examination of the immunostaining pattern in gicerin-positive neurons revealed a characteristic outlining of the somata of stained cells as well as complete staining of the cell processes, including the central and peripheral axons and their growth cones (Fig. 4 and 5). These cellular localization of gicerin in transgene-positive neurons in zebrafish brain were similar to that of gicerin-expressing neurons in the developing chick brain (unpublished data). In embryos containing a large number of cells expressing transgene, gicerin-positive neurons showed a fasciculation formation between their contacting central axons, resulting in a specific path of axonal tract, while their peripheral axons were in a form of nonfasciculated arborizations. A representative transgenic embryo was demonstrated in Fig. 5. The growth cones of central axons projected from two neighboring gicerin-positive Rohon-Beard neurons in the spinal cord exhibited a beginning of fasciculation formation, by catching and following the preceding axons each other (Fig. 5A). From the double-immunostaining of the same embryo with a HNK-1 monoclonal antibody, which

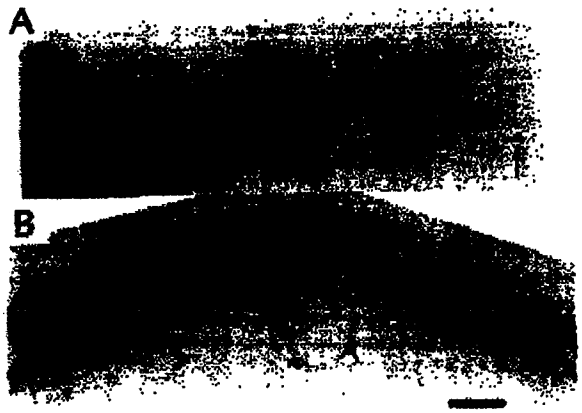

Fig. 5. Chicken gicerin was detected on the entire projection of axons as well as cell bodies in transgene-positive neurons in transgenic embryos stained with an anti-gicerin antiserum in a whole-mount preparation. As an example, two gicerin-expressing Rohon-Beard neurons (indicated as 1 and 2) in the spinal cord of a transgenic embryo were shown (A). They project central axons both anteriorly and posteriorly within the dorsolateral spinal cord, while their peripheral axons are arborizing in the surface of trunk. The growth cones of their central axons (arrowheads for axons projected from neuron 1 and arrows for axons from neuron 2) are catching and following the preceding axons at the sites of contact (asterisks) where they may start to fasciculate and form the longitudinal axon tract. In (B), the same embryo in (A) was double-stained with an anti-HNK-1 antibody which could stain almost developing neurons. Including the transgene-positive neurons, HNK-1 epitope-containing neurons are forming two bundles of dorsolateral axonal tracts within the developing spinal cord. Anterior is to the left, and dorsal is upwards. The scale of (A) is 90% of (B). Bar, 25 um.

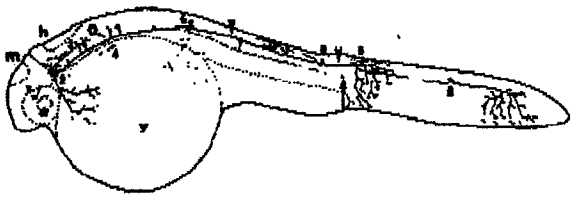

Fig. 6. Schematic diagram illustrating the relative positions and morphology of gicerin-positive neurons in a representative mosaic transgenic embryo microinjected with pNFP-gicerin. Twenty neurons and no non-neuronal cells were detected by an anti-gicerin antiserum in this embryo. Only one side of the body was illustrated for the simplicity. The transgene was expressed in various type of neurons in transgenic embryos at 26 h. Anti-gicerin antiserum stained a neuron (1) located in the region of ventrocaudal cluster in the brain, its long descending axon extended to the half of body length in path of the ventral longitudinal tract (arrow). In parallel with this axonal tract, descending central axons of trigeminal neurons (2) and central axons of Rohon-Beard neurons (5) were arranged in the path of dorsal longitudinal axon tract (arrowhead), while their peripheral axons arborize extensively in the skin. Neurons in the hindbrain (3), in the region of postotic commissure (4) and others are also shown. m; midbrain, h; hindbrain, o; otic vesicle, e; eye, y; yolk.

could stain almost developing neurons, this fasciculation was confirmed as a longitudinal axon tract in the dorsolateral spinal cord (Fig. 5B). It is considered that the formation of a fasciculation between gicerin-positive neurons in transient transgenic zebrafish may partly result from the homophilic cell adhesion activity of exogeneous gicerin.

With the prominent expression of gicerin in the primary sensory neurons (trigeminal ganglion neurons and Rohon-Beard neurons), the transgene was also detected in various types of neurons in the transgenic zebrafish embryos according to their developmental stage; neurons in the region of the ventrocaudal cluster in the brain, hindbrain, otic vesicle, postotic commissure, motoneurons, ascending and descending interneurons, and so on. Among hundreds of gicerin-positive embryos, we illustrated a representative transgenic animal with schematic diagram which shows the relative position and morphology of gicerin-positive neurons (Fig. 6).

DISCUSSION

We firstly found that the murine neurofilament gene promoter is activated in specific regions of developing zebrafish embryos, suggesting that the promoter activity is conserved from fish to mammals. Previously, several groups have studied NFP-deriven reporter genes in different mammalian cell lines, but no cell-type specific expression was observed (14). These results imply that in vitro expression experiments of NF gene do not address the tissue-specificity of NF gene expression. Therefore, experiments were required using transgenic animal in order to examine the neural specificity of NF gene expression (20, 21). Our data using zebrafish are comparable to those of transgenic mice, with regard to the tissue-specificity of NF gene promoter, and support the usefulness of this rapid transient transgenic animal system in the functional analyses of promoter activities that regulate gene expression in vertebrates.

In this transient expression system, the majority of animals contained a small number of transgene-positive cells per embryo, probably due to a mosaicism of transgene expression (3) or its combination with tissue-specificity of the promoter. The restricted pattern of transgene expression, however, is rather favorable for the analyses of the roles of cell adhesion molecules in the developing nervous system, because the expression of transgene only in a few cells is easy to follow overall development of the neurons in transgenic embryos.

We used the NFP to direct ectopic expression of chicken gicerin to the nervous system in zebrafish. Tran-

sient transgenic embryos injected with the NFP-gicerin construct showed somewhat more neuron-specific pattern of expression than those injected with NFP-lacZ construct. This is probably due to difference in sensitivity of the assays used to detect transgene expression or promoter strength between nervous system and the other tissues. The other possibility is that the expression of chicken gicerin affected cell differentiation of the developing zebrafish embryos. It has been reported that a switch in cell fate can be induced by ectopic expression of a gene (22,23).

Chicken gicerin was expressed on the entire projections of neurons, including axons and growth cones as well as cell bodies, in transgenic embryos. Axons from gicerin-expressing zebrafish neuronal cells exibited a fasciculation formation with neighboring gicerin-positive axons, may partly due to homophilic cell adhesion activity of gicerin (9,10). Although we did not examine the interactions between chicken gicerin and endogenous molecules in zebrafish, it is possible that the presence of NOF- and gicerin-like molecules in zebrafish can interact with the exogenous chicken gicerin by heterophilic or homophilic cell adhesive activities. Recently, several studies have provided new insights into the cellular and molecular cues required for correct axonal pathfinding (24,25,26). These studies have uncovered the intriguing evolutionary conservation of the basic molecular and cellular mechanisms underlying growth cone guidance and target recognition, since many proteins involved in such process share structural similarities in different species.

In conclusion, our experimental results support the usefulness of the transient transgenic system using zebrafish in the analyses of gene promoter activity, and suggest that transgenic zebrafish system may be very useful in studying the in vivo function of a variety of genes during neural development.

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Exhibit 6

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THE MOLECULAR BIOLOGY OF TRANSGENIC FISH

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THE MOLECULAR BIOLOGY OF TRANSGENIC FISH

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I. Introduction

The production of transgenic fish is hot in biotechnology and research into the molecular genetics of development even though the technology is relatively new. In 1985 a major symposium on the genetic engineering of animals was convened but transgenic fish were not a topic (34) and the Second International Symposium on Genetics in Aquaculture did not have any presentations involving transgenic fish (41). But, that same year Zhu et al. (165) published the first report of introducing growth hormone genes into fish, Gill et al. demonstrated that heterologous growth hormone stimulated piscine growth (42), and the first fish growth hormone cDNA was cloned and expressed (134). An explosion of research reports on transgenic fish soon followed even though gene transfer as part of whole chromosomal material had been demonstrated in fish several years earlier (152). There are three primary reasons why transgenic fish are attracting so much attention. 1) Transgenic fish may play a major role in aquaculture (17,36,53,66,69,95). 2) Transgenic fish are increasingly being recognized as a superior system for examining the molecular genetics of early vertebrate development (98,123,128,133,138). 3) Transgenic fish are under careful political scrutiny with respect to the introduction of foreign and rearranged genetic sequences into fish genomes and the potential of engineered fish to alter specific ecologies and the environmental *status quo* (76,77).

Whether for commercial aquaculture or scientific study, three major steps must be undertaken in order to produce transgenic fish. First, a specific recombinant DNA *construct* must be prepared. The construct has both the gene encoding the protein of interest and the genetic regulatory elements which direct expression in a temporal, spatial, and/or developmental manner. Second, the recombinant construct must be delivered to the nuclei of cells in the developing embryo in order for it to be distributed to all tissues of the fish. Third, since not all deliveries of the transgene will be effective and not all constructs will behave in the manner that was desired, a screening process for those fish with active transgenes must be established. Efficient and accurate procedures for each of the three steps must be available for achieving the myriad of goals that genetic engineers of fish entertain. Developments in all of these areas are proceeding at a rapid pace. In the following sections I will establish some benchmarks that are required for efficiently producing transgenic fish for research and commerce and evaluate the progress towards satisfying these criteria.

II. Construction of Expression Vectors for Transgenic Fish

Genetic engineering of fish for economic or scientific purposes generally requires the ability to express a protein of interest in the desired tissue(s), at the desired time(s)

and at an appropriate level. To achieve these objectives the gene must be cloned into a vector that has the appropriate expression capability. This section discusses the considerations that must be made in choosing or constructing a recombinant DNA for the production of transgenic fish.

1. Plasmids as vectors

Expression vectors are recombinant DNAs that carry the transgene of interest and the regulatory sequences that determine where, when, and the level at which the transgene will be expressed. Fig. 1 shows a diagram of the general features found in all expression vectors used in transgenic fish studies. All of the vectors reported so far are plasmids that can be replicated to levels of 500-2000 copies per *Escherichia coli* cell. Since *E. coli* can be grown easily to 2-5x10^9 cells/ml of culture broth, one milliliter of bacterial cells can produce more than 10^{13} recombinant plasmids/ml. The ability to obtain practically unlimited copies of recombinant DNAs in relatively pure form makes transgenic work possible.

2. Transgenic DNA

For many purposes the plasmid vehicle is removed from the DNA that will be transferred to cells. For commercial purposes transgenic fish will not have plasmid sequences since these are of prokaryotic origin and accordingly are not useful to the fish. Moreover, the regulatory requirements governing the release of transgenic organisms may be simplified when the genetic material transferred into the recipient cells is not evolutionarily distant to the host DNA. For this review transgenic DNA is the recombinant construct with or without the vector; the transgenic DNA will always have the transgene encoding a particular protein and the appropriate transcriptional control sequences.

a. Genetic regulatory sequences

Cellular DNA has two primary functions, to encode proteins which catalyze biochemical reactions or to serve as structural elements and sequences which regulate the transcription. Current estimates suggest that vertebrate genomes contain approximately 100,000 genes of which about 12,000 are expressed in any given cell. About 10,000 genes serve as housekeeping genes that are required for simple cellular functions, leaving about 2,000 genes that permit each cell to perform its specialized functions in a particular tissue (64). Consequently, in most cells, most genes are off most of the time and are only activated for expression in response to precise signals. Genetic engineering of fish requires an appreciation of the use of DNA as a regulator of gene expression as well as a source of information specifying protein sequence.

Genetic regulatory elements are sites required for initiation of transcription, termination of transcription, RNA splicing and initiation of protein synthesis. The genetic elements regulating initiation of transcription will determine when, where and extent to which expression will occur. Consequently these signals have been been the focus of considerable study by many labs. Transcriptional termination, the generic term for 3' cleavage and polyadenylation, and splicing are important and necessary. But, since they are generally considered to be ubiquitous there has been far less study of how these elements can be used to regulate gene expression in transgenic animals. However, recent evidence indicates that in fact post-transcriptional modification of mRNA by splicing (70,71) and polyadenylation with different poly(A) signals may influence gene expression (129).

b. Regulatory elements: Promoters, enhancers and silencers

Promoters have several definitions but for the purposes of this review the promoter will be considered the DNA sequence that is immediately upstream of the transcriptional initiation site, i.e. within the first 100 base pairs of where mRNA synthesis begins. Other sites on the DNA that are farther away from the mRNA initiation site which enhance or depress transcription are commonly called enhancers or silencers, though depending on the context in which it is placed a single site can be either enhancing or repressing (125). The promoter and the enhancer/silencer sites are *cis*-acting; they affect transcription on the same segment of DNA on which they reside. Generally the proximal promoter region of most genes will direct a basal level of transcription that may be enhanced or depressed according to the availability of *trans*-acting protein factors in the particular cell type (*e.g.*, 28,39,68,106,108). Promoter-distal enhancer/silencer sequences bind *trans*-acting proteins which have at least two domains, one for binding specific sequences of DNA about 6-12 base pairs in length and another domain responsible for specific protein:protein interactions. By binding to the DNA and also binding to other DNA-binding proteins, the DNA can be contorted and nucleosome positioning around genes may be altered (124) to enhance or repress RNA polymerase binding. The binding of one protein may either promote or interfere with the binding of an activation protein and thus lead to enhancer or silencer activity (88). Thus, it is possible that sequences identified as enhancers may in fact reduce transcription (26) and a given *trans*-acting protein bound to a common *cis*-acting site may function either positively or negatively (29,125). Consideration of these points is critical in designing expression vectors for transgenic fish. With these considerations in mind, a rough ranking of the transcriptional power of various enhancer/promoter complexes is shown in

Table I. Since there is not a standard reference, this evaluation depends on comparisons of transcriptional activities from many sources.

Evaluation of transcriptional strength depends on accurate measurement of RNA synthesis which is generally accomplished by either nuclease S1 analysis (94) and/or primer extension analysis (102,103). These two procedures used together are essential for rigorous definition of transcription initiation sites. However, in many transgenic studies the level of mRNA, which is dependent on the relative rates of transcription and RNA lifetime, cannot easily be quantified due to low accumulation rates. Therefore enzymatic activities of reporter genes such as chloramphenicol acetyl transferase (CAT), which can be measured accurately in small amounts, are often used to quantify RNA expression levels. This approach is not rigorous since false transcriptional or initiation sites may subject the mRNA to alternative translation and/or processing and thereby not reflect the true transcription rate. Such effects have been noted in other transgenic systems (49,50,71) though not yet in fish. A further problem with many enzymatic assay systems is that their activities are nonlinear with enzyme concentration; once the substrate is completely converted there is peak activity regardless of enzyme excess beyond this limit. Thus, for all quantitative studies, standard activity curves must be employed to correct the raw data. In most of the publications on relative promoter activities and promoter mapping in fish the displayed CAT conversion levels were in excess of 60%, in the non-linear range of the assay (106), and standardization was not reported. Consequently, the rating of enhancer-promoters in Table I must be viewed with caution since many reports of expression in a variety of piscine cell cultures and/or species were used for its compilation. Where it was possible, CAT activities under 30% conversion were used for ranking since up to this value the enzymatic activities represent enzyme concentration in a linear manner (106).

Transgenic studies are the most valuable for dissecting transcriptional regulatory elements since the transcriptional activities in many tissue types where different *trans*-acting factors are present is possible. In vitro, strong tissue-specific promoters might be scored as weak when measured in an inappropriate cell line. Constitutive enhancer/promoters (*e.g.*, actin, metallothionein, and viral promoters) have generally been used to promote ubiquitous expression, and appear to be the strongest promoters. Careful analysis of transcriptional regulatory elements in transgenic fish (106,108) has the potential to serve an important role in future studies of activation of RNA synthesis, as discussed further in Section VI. These studies are urgently needed for controlled gene expression in a spatial and temporal context.

b. Transgenes

Transgenes can be classified into three groups that are schematically depicted in Fig. 2. The most common is a modified gene that encodes a desirable protein product. Delivery of such a gene or its cDNA (Fig.2-1a,b) will confer a *gain-of-function* to the cells in which the gene is expressed. Transfer of growth hormone (GH) genes, isolated from a variety of sources is the most common example in fish. In some instances fusion genes containing sequences from two genes can be employed for specific purposes (Fig. 2-1c). Table II lists many of the transgenes that confer gain-of-function genes to transgenic fish and the regulatory sequences that drive the transgene. Additionally the completeness of the gene must be considered. For example, the presence or absence of the signal sequence on transgenic GH gene products will affect the level of GH in the blood as well as protein concentrations in different cells. The strength of the promoter may be less important in this context than the state of the transgene.

The most common type of *gain of function* transgene found in the transgenic fish literature are reporter genes (Fig.2-2a) whose functions do not necessarily influence fish physiology but do permit examination of the regulatory sequences that then can be used for applied genetic engineering. Expression of these genes is easy to quantify and/or locate. The two most common genes for these purposes are the bacterial chloramphenicol acetyl transferase (CAT) and *lacZ* (b-galactosidase, b-gal) genes. CAT activity is extremely easy to measure in cellular, tissue, or whole animal extracts (46). b-galactosidase is useful for locating the sites of its expression in tissue slices or whole animal samples since it can convert a colorless lactose analogue (5-bromo-4-chloro-3-indolyl-b-D-galactoside) into a blue indolyl dye by cleavage of the galactosidic bond. Thus, the CAT and neomycin phosphotransferase II (NPT , *neo*) genes are useful for quantifying the amount of expression. NPT has the added advantage that it can be used in both negative and positive selection screens (97). In contrast, the *lacZ* gene is most useful for determining the site or period of gene expression in a complex tissue or animal. These reporter genes plus those for vital dyes are used to evaluate the usefulness of transcriptional regulatory sequences in terms of their power and tissue-specificity (*e.g.*, 153). Table III lists transgenic constructs used in fish that employ reporter genes behind various enhancer-promoter complexes.

The third class of transgene (Fig. 2-3a,b) has not yet been employed in transgenic fish studies, but it will. These are *loss-of-function* genes whose expression will interfere with expression of normal genes. Such genes might encode antisense RNA to interfere with the processing and transcription of normal genes (74) or catalytic RNA (a ribozyme) that can cleave specific mRNAs and thereby cancel their function (63). Alternatively, genes encoding partial protein structures or mutated protein structures, known as

dominant negative mutations, can be used to interfere with the function of normal proteins (65a). Such constructs will have great use for studies of the genetics of vertebrate development in the coming years but probably less use in aquaculture (see sections V and VI).

The transgene itself may be take any of several forms. Small genes are easier to clone and handle than larger genes. Most animal genes and most eukaryotic genes encoding proteins are broken up into exon sequences separated by intervening intron sequences. Following transcription, a precursor mRNA will be spliced into a smaller mature mRNA lacking the introns. In animal cells intron sequences often are 80-90% of the length of gene but may be as much as 98% the length of the gene. For this reason, cDNA copies of the mRNA are often used as transgenes. However, several studies in animals and tissue culture have shown that many (but not all) genes are not sufficiently expressed unless they are transcribed as a precursor containing an intron destined for removal by splicing (10,70). Although all of the reasons for this are not clear, nearly all transgenic constructs are designed to have an intron sequence that can be spliced out. Consequently, an expression vector that includes an intron can function for both intact genes, mini-genes (genes that have had one or more but not all of their introns removed), and cDNAs or processed genes (58).

3. All-fish expression vectors

One of the major applications of transgenic fish is the addition of characteristics that will improve the value of commercial fish (see section V below). Early transgenic fish used transcriptional regulatory elements from land vertebrates and their viruses. These constructs were active in fish cells and suggested that the regulation of gene expression was equivalent in land and aquatic vertebrates. The molecular dissection of the carp b-actin gene and its transcriptional regulatory sequences showed that they were more conserved than either the cDNA nucleotide sequence or the amino acid sequence (94). Since the *cis*-acting elements of the gene must interact with rather promiscuous *trans*-acting proteins, the *trans*-acting factors in fish must be similar to those in mammals and birds. Consequently, in retrospect it is hardly surprising that fish regulatory sequences function in mammalian and avian cells and vice versa (Tables I, II, III). Viral genes are activated by the same cellular proteins that activate nuclear genes. However, owing to their association with dread diseases, the use of viral enhancers and promoters in food fish is beyond popular acceptance even though long terminal repeat-like (LTR) sequences, similar to those found in RNA tumor viruses, have been found in piscine genomes (75,109). Likewise, the use of DNA sequences from land animals, though equivalent in all *biological-functional* respects, is seen often as morally and/or socially

unacceptable. Accordingly there has been an effort to develop "all-fish" expression vectors.

Several groups have developed expression vectors which have fish genetic regulatory sequences for enhancer/promoter activity, intron activity, and polyadenylation. The first vector developed by Liu *et al.* used the carp b-actin gene enhancer-promoter and first intron of the b-actin gene in concert with the poly(A) cleavage/addition signal from chinook salmon (91). This vector came in two major types, one with just the proximal promoter and the second with all of the enhancers including an enhancer in the first intron. Since the first exon is non-coding for all vertebrate actin genes so far characterized, the intron actually precedes the transgene insertion site. The salmon GH 3' end was chosen over the b-actin 3' sequence since the latter has a silencer sequence next to the poly(A) signal that reduces expression in muscle cells. Consequently, these expression vectors are constitutively expressed in nearly all tissues. The earliest form of the vectors had a single *Kpn*I site for addition of the desired transgene. Further developments have produced FV-5 and FV-6 fish expression vectors which have a complete polycloning sequence for insertion of a greater varieties of DNA (Fahrenkrug and Iwan, unpub.).

Hew's lab (28) has constructed all-fish expression vectors based on the antifreeze protein (AFP) gene promoters. The AFP genes are expressed at lower levels than b-actin and their tissue distribution is not as ubiquitous. They have analyzed the expression of AFP genes in various tissues from winter flounder, ocean pout and the sea raven by northern blot RNA analysis to conclude that the first two genes are expressed in many tissues whereas the sea raven AFP gene is expressed only in liver (45). The Hackett, Hew and Schartl labs have extensive collections of constructs based on their respective promoters that came from enhancer/promoter mapping studies. The range in activities, as measured in both tissue-cultured cells (28,39,68,91,92,106,108,155) and fish (29,68, 91,93,106,107,108,154,155) is 100- to 500-fold. Although the AFP promoters are weaker than the b-actin promoters, the transgenic fish containing GH genes behind these promoters have the greatest growth enhancement (29) as discussed in section V below.

The Schartl lab (68, Hong and Schartl, pers. comm.) has constructed several all-fish expression vectors based on promoters from the two rainbow trout metallothionein genes tMTa and tMTb. These constructs can be induced 2 to 100-fold following induction with heavy metals, depending on the cells or tissue and the metallic inducer. Induction with zinc produces about half the transcription of the carp b-actin construct with enhancers (Table I). This vector should be extremely useful for many studies of gene regulation in fish since it is inducible and can be regulated. Bayer et al (4) reported

the construction of an expression vector with the ependymin promoter that shows ependymin-specific temporal and spatial expression patterns using the b-galactosidase marker gene. Together with the development of heat shock promoters by the Hackett and Westerfield labs (91 and pers. comm.), these represent the initial forays into tighter regulation of transgene expression.

Currently most of the regulatory sequences used to drive transgenes are constitutively acting; they are switched on in nearly every cell although there may be some variations, *e.g.*, the RSV LTR is most active in fibroblasts whereas the SV40 enhancer is most active in epithelial cells). Development of finely tuned genetic regulatory sequences is essential for both commercial and scientific exploitation of transgenic fish. **As more fish genes are found, the number of transcriptional elements that can be incorporated into vectors will increase. Studies in vertebrates indicate that practically any enhancer-silencer can be coupled to any transgene for expression (81).**

NOT through random Integration

III. Methods of transgene delivery

Methods of gene transfer that have been successful with transgenic mice have been adapted to fish. However, methods that are reasonable for mouse eggs, which are precious and require a substantial amount of work to orient, inject, and reimplant in a pseudo-pregnant female mouse, are not necessarily ideal for fish embryos which are abundant, large, often available once a year, and have harder chorions. Moreover, to produce transgenic lines of fish, successful transfer and integration of the transgenic DNA into zygotic chromosomes of either the first cells or those leading to germ-line decent is necessary. This is a significant problem in fish, where most transgenic fish are mosaic, as compared with murine embryos. Accordingly, methods that are sufficient for a few dozen mammalian embryos are not necessarily the best for thousands of fish embryos. Five techniques of gene transfer, microinjection, electroporation, sperm delivery, ballistic, and lipofection, are considered next.

1. Microinjection

Microinjection is the most popular form of gene transfer into fish. The techniques were developed using mammalian embryos which were obtained in relatively small numbers, about 10-30 eggs depending on the species and the degree of superovulation (14,25). Essentially the eggs were bathed in a saline solution, the pronuclei were injected with about 1 nl of DNA solution, and the embryos reimplanted in pseudopregnant mice. The rate of successful transfer and integration of DNA is about 25% for mice in a good transgenic mouse facility but may be much less for other animals (59,118; S. Hughes, per.

comm.). Microinjection is relatively efficient with mouse eggs since the cells are small, the pronuclei are visible, and the eggs are not surrounded by hard shells.

Fish eggs vary in size from 1 to 7 mm diameter, about 300-30,000 times larger than a mammalian egg; and the ratio of cellular/nuclear volume in a mammalian egg is about 20 whereas is more than 100,000 in a fish egg. Thus, with such greater volume, localizing the pronuclei in teleost eggs is quite difficult. Additionally, the rapid onset of cleavage and the hardening of chorion following fertilization interfere with visualization of the pronuclei in fish eggs and rapid injection of transgenic DNA. Nevertheless, since microinjection is such a straight-forward procedure (25), a variety of attempts have been made to adapt the procedure to fish eggs (36).

We (56,162) and others (36,165; Z. Zhu, pers. comm.) have tried chorion removal without much success; the dechorionated eggs are not able to survive past a few days following chorion removal. However, there are some reports of successful removal of chorions from model fish (22,128,140,141) but the embryo survival and transgene integration rates are not much better than those obtained without chorion removal. Not all investigators employ dechorionation techniques after considering the work involved to determine a reliable protocol that is effective for a given type of egg. Microinjection through the micropyle has been accomplished by Hew's lab for Atlantic salmon eggs but the technique is quite difficult to master (C. Hew, pers. comm.) and by Moav and Maclean for tilapia eggs (71a, 124a). For hard chorions, success has been achieved by drilling a small hole in the chorion before insertion of a brittle glass needle (54,126) or by depending on steel or hard needles for penetration. We have found that for injection of walleye, northern pike, salmon and trout eggs, direct injection is generally the most efficient method (107). Though the breakage of needles is relatively high, there are fewer uncertainties and variations in the procedure which leads to more reliable survival rates. Direct microinjection through the relatively soft chorions of zebrafish, catfish and other species is clearly easier and the preferred method for some. However, both Stuart *et al.* (140,141) and Culp *et al.* (22) dechorionate zebrafish embryos; the former group reports a 4-5% integration rate of transgenic DNA while the latter group has an 17% average integration rate. Both labs use a mild pronase treatment of the chorions but their methods of analyzing for transgenic fish differed. Whereas Stuart *et al.* used an expression assay for their CAT reporter gene, Culp *et al.* employed polymerase chain reaction (PCR) to detect the presence of expressing as well as silent transgenes in their test fish. PCR is far more sensitive than the enzymatic assay.

Localization of pronuclei by dye injection has met with limited success (59,158) but the effects on the developing embryo and its genome are unknown. Consequently,

most investigators aim for the embryonic cytoplasm for delivery of 1 to 2 nl of DNA solution containing 10^5 to 10^7 molecules of DNA, which might be expected to be a problem to a cell or nucleus that has practically no free H_2O (40). Piscine nuclei are on the order of 0.05 nl in volume, about 2-10% the volumes injected. Zygote cytoplasms being much larger could presumably tolerate the influx of water more readily. The effects microinjection of DNA into 1- to 2-cell stage embryos on embryonic survival and DNA integration into the piscine genome are shown in Fig. 3. Although increasing amounts of DNA (up to $>10^7$ copies injected) tend to improve integration rates (Fig. 3B), it is at a cost of survival (Fig. 3A), which may be due to insertional mutagenesis or the unusual presence of so many polyanions. Surprisingly, there is no correlation between cell mortality and volume of fluid injected in the ranges used for salmonid transgenesis (Fig. 3C) even though the presumed volumes of injection were sometimes in excess of 100 nuclear volumes. The scatter in the points in Fig. 3C is due to different concentrations of DNA used by investigators in their experiments. In their review, Fletcher and Davies (36) found a wide variation in survival and expression results. However, much of the variation can be attributed to learning the subtle tricks of microinjection, since different groups attempted alternative techniques with variations on common themes in the early years of fish genetic engineering before reliable protocols were developed. In considering the early literature it should be noted (see below) that the early criteria for determining integration rates was not rigorous.

In summary, the following can be concluded from the accumulated experiments that are listed in Tables I-III. 1) Embryonic survival for most species following microinjection is 80-90% that of uninjected controls when less than 80 pg ($<10^7$ copies of transgenic DNA) were transferred; the outcome of most experiments depends on that elusive characteristic, *egg quality*. 2) As discussed in more detail in section IV below, microinjection generally leads to about 50-80% of the eggs being able to express the transgenic DNA shortly after gene transfer but the persistence of high levels of expression is not maintained over the generation time of the fish and this remains a problem. 3) Nearly all (90-99%) of fish that have integrated transgenes will be mosaic for its presence and/or level of expression, suggesting that integration of the injected DNA was not efficiently occuring in the 1-cell stage of embryonic development (22,54,57,124a,131,141,164). Even zebrafish which have a relatively high rate of integration, ca.5-20%, are generally mosaic (22,140). These relatively high rates of mosaicism and low transgenic level are compensated by the large number of eggs that can be microinjected by the accomplished investigator. After training, rates of injection of between 50 and 1000 eggs/hr, depending on the type of eggs, the logistical support, and

the accuracy of injection, can be achieved. These results serve as benchmarks against which to compare other gene transfer techniques.

Fish of commercial importance such as salmon, trout, carp catfish, northern pike, and walleye spawn once per year and deliver 800 to 100,000 eggs/spawn. These eggs undergo their first cleavages in 30 min to 15 hours (36). Although eggs and sperm can be saved for days by refrigeration, there is relatively little time for injection of massive numbers of embryos if egg and milt quality is to be preserved. Injection rates of 1000 eggs/hr (B. Moav, pers. comm.) though amazing are still insufficient when successful production of germ-line transgenic fish is a few percent or less. Consequently, there is a major drive to find more efficient procedures for mass transfer of DNA to thousands of eggs at once. The following four techniques, electroporation, DNA-binding to sperm, particle bombardment, and lipofection, are being investigated.

2. Electroporation

Electroporation is a method that has been successful for gene transfer to prokaryotes and eukaryotic cells in tissue culture (113,122,136) and other materials into fish embryos (160). Since 1990 electroporation has been finding greater favor for transgenesis in fish. Inoue *et al.* (73) demonstrated that electroporation of an mMT/rtGH construct into medaka resulted in 25% survival of the eggs to the hatching stage with 4% of these being transgenic in their germlines. Though the survival rate was low, to obtain 31 transgenic fish from a few electroporation trials using several hundred embryos per electrical shock was impressive. Inoue's experiments utilized 800 ml of solution containing 80 mg of DNA per shock, or about 100 ng DNA/embryo; capacitor-driven, 50 msec-pulses at 750V/cm were used. Alternatively, electroporation of zebrafish with an RSV/CAT construct yielded 75% survival with a 65% rate of DNA uptake with triple 0.1 msec pulses of 125V/cm for batches of 200 eggs in phosphate-buffered saline containing 100 mg DNA/ml (13). Buono and Linser (13) found that Wescodyne@ at 0.1% used for germicidal purposes was in fact a key ingredient to permeabilize the chorion to DNA. This report claims successes and failures by others using their protocol. Although our earlier work with dechorionated goldfish embryos was not successful (Hallerman, unpub.), we have had success with intact zebrafish embryos following the protocols described by Buono and Linser (13). We have found that each electroporator demands tweaking of the controls for optimal responses; Breuer (per. comm.) has determined that triple pulses at 120V/cm reproducibly results in 80-90% survival with an impressive uptake of several thousand copies of cb-act/CAT DNA/fish. Baekon, Inc. advertising claims a 90% survival of zebrafish subjected to their procedure with a 40% integration rate, based entirely on positive Southern hybridization of isolated DNA, a non-rigorous

test for integration but sufficient for uptake studies. However, Baekon's negative results with electroporated zebrafish embryos is not consistent with the results cited above; the machine is probably less important than the time spent in making the procedure work under local conditions. In conclusion, considering the variation in capacitor ratings, the results over the past two years are consistent and promising. We can expect that the results will vary for the eggs of different species of fish.

An interesting variation on the electroporation technique, electroporating sperm prior to fertilization, has been developed by the Orban lab and tested in collaboration with the Maclean lab (111). They used carp, African catfish and tilapia as their experimental systems since obtaining sperm from larger fish is relatively easy, their sperm are less refractile to handling and each fish yields greater quantities of sperm which are motile for several hours. They demonstrated with tk/neo and pGM3H4/CAT transgenic constructs that 5-20 ml of semen containing about $5x10^7$ to 10^8 sperm electroporated at field strengths of 750 to 2250 V/cm yielded a 70-80% fertilization rate compared with controls and a decrease of 10-30% in motile sperm. Although only 2-4% of the eggs were positive in terms of DNA uptake or NPT activity, these low results represent only the beginnings of an aternative method of mass gene-transfer which could avoid the "bottleneck" in current procedures (53).

3. Sperm Carriers

With the spectacular report of Lavitrano (87) that mouse sperm could be used to convey externally bound transgenic DNA into recipient eggs, lead many transgenic fish groups to attempt the same procedure with different species of fish. Indeed, we tried this procedure a year before the report in mice was published and found that [^{32}P]-labelled RSV/neo DNA could be bound to sperm and taken into eggs; but there was no evidence of expression (Yoon, unpub.). H.-W. Khoo (unpub., pers. comm.) has reported that sperm-mediated transfer of mMT/hGH into zebrafish eggs can successfully result in transient expression of hGH (determined immunologically) from transgenic constructs that were often fragmented and extrachromosomal. Although DNA-binding to sperm has been demonstrated by several teams, no one except Khoo has presented any evidence of successful sperm-mediated transgenesis in fish (2,3,16, reviewed in 36). There was no evidence of chromosomal incorporation or expression in the F_1 progeny of founder fish. Although the Lavitrano mouse data is not reproducible (12), the idea spawned the procedure of electroporating sperm so that the internally-confined transgenic DNA could be carried into the unfertilized egg without as much injury (111). The method may also work if the transgenic DNA is encapsidated in a viral coat; for instance, recent evidence suggests that infectious hematopoetic necrosis virus may be transmitted vertically via

binding to sperm (110). Thus, transduction of transgenic DNA encapsidated in a viral coat may be an alternative means of using sperm to carry the construct into the embryo at the single cell stage.

4. *Particle Bombardment: Gene Guns*

High-velocity microprojectiles have successfully permeated cell walls and membranes to convey nucleic acids into living cells (82). The method is especially suited for plant cells which have thick cell walls that restrict conventional transformation procedures used for animal cells. The procedure has been adapted to fertilized loach, zebrafish and rainbow trout eggs by Zelenin et al. (163 and pers. comm.) using RSV/*lacZ* and SV40/neo as reporter gene constructs. The initial results indicate that three days following bombardment 70% of the embryos from all three species survived and about 5% were reported to exhibit NPT or b-galactosidase activity. Embryos shot with SV/neo exhibited a surprisingly high survival rate in water containing the neomycin analogue G418 at 600-800 mg/ml. 20-40% of the developing fish survived for 6 to 9 days, depending on duration and G418 treatment. Eventually, after two or more weeks, most of the fish died, presumably because not all tissues continued or were able at all to express NPT activity. As we have shown previously, G418 is a powerful screen for mosaicism and either the transfer and immediate integration efficiency must be high or the number of embryos must be enormous to overcome low survival of the experimental fish (161). The procedure might be much better used on F_1 progeny where the numbers surviving would be higher due to decreased mosaicism in true transgenic offspring.

5. Lipofection

Another procedure that has been successful with nucleic acid transfer in tissue culture is lipofection where in synthetic lipid vesicles encapsulate nucleic acids or nucleic acid:protein complexes and permit their uptake into cells following fusion of the vesicle with the plasma membrane (*e.g.*, 96). Following on the work of Szelei and Duda (144) in which high molecular weight DNA could be entrapped by phosphatidylserine, containing 25-50 mol% of cholesterol to increase vesicle size and to stabilize the liposomes in the presence of protein, dechorionated catfish zygotes at the 2-4 cell stage and 4-16 cell stage were subjected to lipofection (145). The substrate nucleic acid for delivery was recombinant lambda phage DNAs, containing either the tumor necrosis factor (TNF) gene or the human GH (hGH) gene, packaged in phage head protein. The addition of protein apparently increases the efficiency of entrapment by the liposomes and protects the integrity of the recombinant DNA. Optimal transformation was achieved with a stoichiometry of 2000 liposomes (700 phage heads) per cell Depending on the liposome/embryo ratio, transformation frequencies resulting in transgene expression

ranged from 60 to 80% in the short term but after a year expression was lost. These data emphasize an important observation, delivery of transgenic DNA is only one half of the problem, the second half is to achieve rapid integration that leads to stable dispersal of the construct to all cells in an expressible state. The second half of the problem is discussed in the following section.

IV. Transgene Persistence and Integration: Effects on Expression

Although millions of copies of transgenic DNA are delivered by any of several techniques to recipient fertilized eggs, only a low percentage of these nucleic acid molecules takes up permanent residence in the fish genome. Most of the results for evaluating expression are based either on transient expression of unintegrated linear concatamers or supercoiled forms of transgenic DNA in embryos following gene delivery. However, both the presence and the state of the transgenic DNA changes over time. Furthermore, in subsequent generations transgenes that were expressed in the parents are often inactive in offspring. The factors which affect transgene expression following delivery into the recipient embryo are discussed in this section.

1. Persistence of Transgenes

Of the millions of copies of DNA delivered per zygote, most are lost while only a few are replicated in either an autonomous state or in an integrated form during chromosomal replication. Transgenic DNA is generally delivered in one of three fashions, 1) as supercoiled plasmids containing both the bacterial vector and the eukaryotic transgenic construct, 2) as linear DNA sequences with or without the accompanying bacterial vector, and 3) as ligated concatmers of linear transgenic DNA without the bacterial vector. There are advantages and disadvantages to all procedures that have relative importance according to the nature of the experiment.

a. Linear vs. supercoiled forms of transgenic DNA

In general, transgenic animals for aquaculture will not have the bacterial vector, for reasons discussed in section II, and thus supercoiled plasmids are not used for this purpose. The form of the DNA, supercoiled or linear, did not affect embryo viability in most studies. Examinations over the first 48 hours following microinjection show that the linear DNAs and supercoiled forms are expressed at about equivalent rates (18,106,107,120,154,155) but thereafter the supercoiled forms give higher rates of expression for the next several weeks due to their relative longevity in an extrachromosomal state. In mice linear, concatemerized molecules were more effectively incorporated into into chromosomal DNA (11). In fish this finding was supported by experiments with injections of SV40/hGH (19) or mMT/tGH into rainbow trout zygotes

(120) wherein linear DNAs gave 75% transformation vs. 40% for circular molecules. However, the assay for integration was the formation of high molecular weight DNA which is an insufficient assay, as discussed below.

Supercoiled DNA can be converted into high molecular weight multimers with and without rearrangements of sequences (18,57,107,154). Linearized DNAs commonly are found in high molecular weight, tandem arrays within minutes after microinjection whether they were delivered as monomers or concatenated polymers (18). These concatemers can be reduced to shorter fragments following restriction nuclease digestion. Owing to head-to-head, head-to-tail, and tail-to-tail association as well as nuclease activity before or after concatemerization, fragments of varying sizes are seen on Southern blots of isolated DNA (18,54,57,101,120,140,165). In the early years of transgenic fish analysis, the presence of high molecular weight foreign DNA and/or unusual DNA fragments, detected by Southern blotting, was taken as an indication of chromosomal insertion. Since the million copies or so of the transgenic constructs can ligate to form high molecular weight sequemces, today more rigorous proof of integration, such as persistence in the second generation, is demanded.

b. Persistence of expression of transgenic DNA

In several experiments done in zebrafish using the cb-act/CAT construct (106), medaka using RSV-SV40/CAT and CMVtk/CAT constructs (39,154), rainbow trout with mMT/tGH (120), and northern pike with the RSV/bGH construct (107), the supercoiled forms persisted at higher concentrations than linear forms for several weeks following microinjection. In zebrafish, approximately 90% of the transgenic supercoiled DNA was degraded while 99% or more of the linearized DNA was destroyed beyond detection by Southern hybridization after ten days (106). Consequently, for studies of transcriptional regulatory motifs, the supercoiled constructs have been analyzed more than linearized versions.

One indication of integration of foreign DNA is its physical persistence into the F_1 generation. However, the report of Stuart (141) showed that extrachromosomal DNA could be transmitted to the F_1 progeny. Thus, although inheritance shown by single-step vertical transmission is necessary for an integrated transgene, it is not sufficient for proof. Unfortunately, only sequencing of junction fragments of transgene and chromosomal DNA rigorously proves integration. This can be done by inverse PCR (114) with relatively little pain for a few samples, but the appeal of transgenic fish is the enormous number of events that are sought so that even this procedure is beyond convenient assay. In the long run, inheritance analysis (section 3 below) is the most practical method for demonstrating integration in the chromosomes of germ-line cells.

Though integration is necessary for continued germ-line transmission of transgenic DNA, the problem of mosaicism and the oft noted lack of transgene expression in the F_1 population remain paramount. More than 95% of transgenic fish are mosaic indicating that integration occurred after the first cell stage of embryogenesis. The steady decrease in transgenic DNA with time is most probably due to random nuclease action plus a low rate of cell death from occasional insertional mutagenesis (84) that may not affect the viability of the whole organism and thus not be readily detectable. In mice the mutation rate is about one mutation per 20 integrations (47,118). The rate in fish is unknown but is presumed to be in the same range. The second problem, the silencing of transgenes following passage into the F_1 and beyond, has been noted by several groups (22, P. Gibbs, pers. comm.). The cause of this is not known but suspected to be methylation during gametogenesis and post-gametogenesis (137,142,143). Swain *et al.* (143) have speculated that paternal imprinting effect may be an easily damaged or "leaky" property of the transgene. Given the enormous number of potential sites for integration, it may be that there are classes of chromatin that are preferred sites of integration and that methylation of these sites occurs frequently during vertical transmission of the transgene. To circumvent this potential action, consideration is being given to flanking transgene constructs with "border elements", also known as nuclear matrix attachment regions and specialized chromatin sequences, that may shield the transgenic sequences and dampen position effects on transcription by adjacent chromosomal regions (8,33,80,121). When large chromosomal fragments are transferred to rainbow trout (27) or complete genomes inside embryonic cells are transplanted to recipient embryos (67a,85,90), expression is maintained. The continued expression of such transgenes is presumably due to their position in their natural chromosomal environment.

Enhancing the temporal integration rate is as important as improving the efficiency of integration. One initial, unsuccessful foray to enhance integration (65) was by using repetitive fish DNA sequences (23,65,148,149) to stimulate homologous recombination by adding an *Alu*I repetitive element to the transgenic construct. Since the repetitive sequences are often tandemly repeated and in transcriptionally-silent heterochromatin, it is not clear that the transgene would have been expressed due to chromosomal effects as discussed above (65). However, use of repetitive sequences for gene mapping of transgenic constructs integrated into chromosomes (112) should be developed.

At least two laboratories are investigating the possibility of accelerating integration with recombinase enzymes (including retroviral integrases and transposases) from eukaryotic and prokaryotic sources. Retroviral integrase and at least some other

recombinases can cleave chromosomal DNA and catalyze integration of exogenous DNA (21,78,79). Mu-recombinase transfected into plant protoplasts can excise and catalyze transposon recombination (60,99) and the yeast *FLP* recombinase can mediate site-specific recombination/integration and gene activation in both insect and mammalian cells (43,115). We have early indications that recombinases can effectively mediate the integration of foreign DNA into fish chromosomes (Izsvak and Ivics, unpub.). In order to have sufficient quantities of the enzyme available for repetitive experiments, we have used the cloned Moloney murine leukemia retroviral integrase (IN) protein in a baculovirus/*Sfi* insect cell expression system (gift from R. Craigie) to produce large amounts of IN. One problem was that resolubilization of IN was difficult following large-scale manufacture (Ivics and Izsvak, unpub.). The Hopkins lab is also working on such a strategy (N. Hopkins, pers. comm.). Since retroviruses are highly efficient at inserting their reverse transcribed genomes into animal host chromosomes, employment of integrases should be extremely useful once the technical problems are solved. Indeed, one of the telling indications of success in this area will be massive death of embryos from insertional mutagenesis when doses of 10^6 to 10^7 copies of transgenic DNA are delivered into single-cell embryos.

Interestingly, should recombinase-mediated early integration become more efficient by several orders of magnitude, the problem of mass transfer will become an even more important bottleneck in the production of transgenic fish. Since sperm-mediated delivery and electroporation are untested for co-delivery of DNA:protein complexes, the strategy of using lipofection capable of co-delivery of DNA: protein aggregates becomes increasingly attractive.

3. Transmission of Transgenes to Progeny

For the establishment of transgenic fish lines, stable integration of the constructs is mandatory for continued vertical transmission to subsequent generations. Although Stuart *et al.*(141) have presented evidence that extrachromosomal DNA may persist into the F_1 generation, an analysis of the rate of transmission of the transgenes to the F_1 and especially F_2 generations indicates true incorporation of the constructs into the telost genome. Table IV relates the results of several experiments where the rates of transmission of transgenic DNA to offspring were examined. In backcrosses of putative germ-line transgenic fish with control fish, a 50% transmission rate is expected. In fact the rates of passage were only around 20% in most experiments (except for that in medaka where it was 100%, possibly due to an enormous number of transgenes integrating into several chromosomal loci). The salmon injected with the antifreeze protein constructs displayed nearly ideal inheritance patterns suggesting that the

incorporation of the transgene did not have any serious effects on viability during development. The lower than expected rates for the other constructs can be due to both mosaicism of germ-line cells (see discussion by Stuart et al., 140) or some of the putative germ-line transgenics really having transgenic episomes. Backcrosses of the F_1 generation with controls should yield an F_2 population with the transgene in 50% of the offspring. This is seen in the zebrafish results (22). Thus, although transgene transmission from the F_0 to the F_1 generation may not be informative, transmission from the F_1 to the F_2 population is a reliable guide for transgene integration in germ-line cells. The incredible 88% passage of mMT1/tGH to the F_2 medaka population (73) can be reconciled if many chromosomes contained a transgene; detailed Southern analyses, in which many specific junction fragments should be evident, were not reported.

These results do demonstrate that germ line transmission from a low percentage of microinjected fish is possible. With the advent of polymerase chain reaction (PCR) techniques, screening of F_1 and F_2 progeny can be done easily and relatively inexpensively at an early stage (*e.g.*, 29). With improvements in transfer and integration procedures, the task would be much easier. Screening could be further simplified if sperm were screened by quantitive PCR to determine the degree of moscaicism of transgenic sperm. Additionally, if the problem of expression in the F_1 generations can be solved; the *neo* screening assay (161) could be employed on the F_1 population to select transgenic fish without the complications that mociasm brings in the founder fish. An alternative positive selection method could employ another set of selectable markers, the barnase/barstar genes (61). The genes for both barnase, the extracellular RNase of *B. amyloliquefaciens*, and barstar, its specific intracellular inhibitor, have been cloned. Expression of barnase is lethal, but co-expression of barstar counters the lethality. Accordingly, barnase under the control of a male-specific promoter could be used to make transgenic fish that would yield only female offspring. Further transgenic constructs with a gene of interest and the barstar gene under either a male-specific promoter or constitutive promoter could yield male offspring that would have the second transgene as well as the barstar gene. Constructs employing barnase could be used for control of transgenic fish and thus could have uses in aquaculture as well as research.

V. Transgenic Fish in Aquaculture

The initial drive for transgenic fish came from attempts to enhance production of economically-important fish. The United States Department of Agriculture (62) along with other world-wide agencies have observed a leveling off of world-wide fish production. With an increasing population, there will be considerable pressure to

increase fish production. What can transgenics offer? Presently there are four areas of preliminary investigation: growth enhancement, cold tolerance, disease resistance, and phenotypic marking. Progress in these areas is evaluated in this section.

Growth enhancement.

Improving the growth rates of fish was one of the initial motivations for genetically engineering fish based on the findings that mouse size could be significantly enhanced following incorporation of a rat growth hormone gene into the mouse genome (119). Three aspects of fish growth could be improved for economic purposes: 1) initial growth rate such that they reach maturation earlier, 2) enhanced growth rate as adults to provide larger fish for market, and 3) fish with improved feed efficiency. In summary, enhanced fish growth rates show considerable promise. Reports up to 1991 demonstrated that carp, salmon, northern pike, loach, trout, and catfish could be transformed with a variety of growth hormones under the control of different promoters to produce fish with growth enhancements of up to 100% that of controls (1,29,48,66,164,166; Z. Zhu, pers. comm.).

Several problems existed with these studies. First, many of the transgenic constructs or growth hormone genes are publicly unacceptable. The use of viral or heavy metal-inducible promoters (*e.g.*, RSV LTR, MT, *etc.*) leads to associations with disease and or metal toxicity (leaded fish). A second problem is how applicable the results obtained under indoor lab conditions will be to outside rearing. In nearly all cases except those in China, the fish are raised indoors under artificial conditions that normally are not used for commercial aquaculture and not optimal for fish rearing to maturity. Consequently, growth rates of transgenic fish with GH genes may vary considerably depending on the facilities available to different research groups. Indeed, the effects of the facility may be greater than those of species variation, enhancer/promoter choice, or source of growth hormone gene/cDNA.

A quantum level leap has been recently reported by Hew's group (29) in which Atlantic salmon embryos microinjected with an ocean pout-enhancer-promoter/chinook salmon-GH cDNA construct yielded transgenic fish that were two to six-fold larger than controls and one fish that was 13 times the average of fish raised from non-microinjected eggs. The fish for this study were selected on the basis of their size and presence of the transgene in their nuclei. Interestingly, the two largest fish died. This observation parallels our findings with northern pike raised from cells microinjected with cb-actin/csGH cDNA in which the largest fish in this study that had germ-line transgenic GH cDNA also died (see Table IV). Also of interest is the all-fish expression vector that was employed by Du et al. (29). The opAFP promoter is not particularly strong. Indeed, the

opAFP enhancer appears weaker than the carp b-actin enhancer, MT promoters and RSV LTR's (Table I) that were used by others. These results suggest that either the strongest consitutively-acting promoters may not always be the best for achieving particular phenotypes, that the Hew group has a particularly fine environment for raising contained fish, or both.

Owing to environmental concerns (76), further treatment of growth enhanced fish will probably be required. In addition to particular rearing facilities such as those at Auburn University (R. Dunham, pers. comm.), the fish will probably require sterilization to provide added security against unexpected escape. Treatments to induce sterility (genetically with the barnase/barstar genes, or by pressure/heat shock) and/or other genetic manipulations could also provide further phenotypic advantages as well as warranties against accidental release. Curiously, the increased mortality the larger transgenic salmon and pike in a couple of growth trials suggests that unbalanced levels of growth hormone may reduce fitness in transgenic fish. If so this would have significance in terms of fears of accidental escape of genetically engineered *superfish.*

2. Cold Tolerance

It is cold in Canada and in Minnesota. To develop an aquaculture industry in such harsh climates, fish with tolerances against cold would be useful and economically beneficial. These considerations led the Fletcher, Davies, and Hew groups to collaborate on the isolation, cloning and transgenic expression of antifreeze protein (AFP) genes found in cold water fish such as the winter flounder(wf), ocean pout(op), and sea raven(sr) that permit survival in freezing seawater, -1.8°C (37,135). The genes and their regulatory elements show evolutionary homology (24,67).

Using CAT reporter gene constructs with wf, op and sr AFP enhancer/promoter, the *cis*-acting transcriptional elements were mapped (45) and the tissue distribution and locus-specficities for enhancer activities were determined by Northern analysis of cellular mRNAs (44). The usual patterns of mixed enhancer and silencer motifs in the 5'-flanking sequences were found in the wf and op AFP Type III genes. However the sr and type I wf AFP promoters exhibited surprising results; the srAFP promoter exhibited 40% of maximal activity without the TATA motif that is involved with RNA polymerase TFIID binding and the Type I wfAFP promoter had basal activity whether sequences between -51 and -2300, including a CCAAT motif, were added (45). Though provocative from a molecular biology vantage, the usefulness of the op promoter, the least unusual in terms of identified transcriptional control sites, is underscored by its effectiveness in driving the salmon GH gene to promote growth (section V-1 above). Atlantic salmon with transgenic AFP genes have been produced with their wfAFP construct (135). 3% of the

zygotes microinjected showed expression of the gene. When two of these founders were backcrossed with control fish, 40% of the offspring carried the gene as detected by polymerase chain reaction (PCR) and 53% of the F2 generation produced by backcrossing F1's with wild type fish showed evidence of the gene. However, expression of the gene was not reported and the fish do not show a measurably increased tolerance to cold (C. Hew, pers. comm.).

3. Disease resistance

Diseases, especially viral diseases can be disastrous to a commercial aquaculture facility. Infectious hematopoetic necrosis virus (IHNV) is a fish rhabodovirus that has caused extensive mortality in northwest Pacific trout and salmon hatcheries. Two approaches have been undertaken by J.C. Leong and her collaborators to find therapeutic measures that will improve virus resistance. The 66 kD glycoprotein (G) gene of the virus has been cloned and expressed in a baculovirus vector (83) to provide large amounts of the protein for passive inoculation by dipping the fish into water containing the G-protein in order to stimulate antibody production against the antigen and thereby achieve immunity (J. Leong, pers. comm.). The viral G-protein has also been cloned as a fusion protein in an *E. coli* expression vector for epitope mapping and protein characterization (158). However, effective immunity on a vast scale might be achieved by transfer of the G-protein gene into the teleost genome for expression in all cell membranes to prevent infection. Accordingly, both the complete gene and a partial gene have been cloned into the carp b-actin expression vector (Fahrenkrug, unpub.) for delivery into trout and salmon eggs (Anderson and Leong, unpub.). These experiments could prove to be more valuable to the aquaculture industry than growth enhancement and cold tolerance. Moreover, from an ecological perspective, the inherent danger of such a genetically engineered fish is relatively minimal.

An alternative strategy for disease resistance would be to incorporate genes into the piscine genome that would nullify viral activity once the virus penetrated cellular membranes. By employing anti-sense gene constructs (74,151), wherein a critical viral gene is oriented backwards to a constitutive promoter, an RNA would exist in cells that could hybridize and completely block expression of the invading viral gene. Use of anti-sense RNA is considered in section VI below.

4. Phenotypic markers

Development of a quick visual screening procedure for transgenic fish would be beneficial in two ways. First, pigmentation of the fish would be convenient and the tyrosinase gene responsible for melanin formation is an obvious candidate (5) though a variety of coloration mutations are known in the zebrafish (Westerfield, pers. comm.).

Work on identifying the zebrafish tyrosinase gene, and others, is ongoing (D. Grunberg, pers. comm.). Second, phenotypic marking would permit easier inspection of fish outside transgenic fish rearing facilities for detection of accidental release of genetically engineered animals. Readily detected transgenic fish might help ameliorate environmental concerns (76). The first report of pigment marking of albino zebrafish employed production of germ-line chimeras using cell transplants from genetically pigmented embryos to embryos from pseudo-albino parents (90). The technique is based on previous work done in mice where HPRT-deficient mouse embryos were produced via embryonal stem cell transplantation (67a,85). Thus, transfer of whole cells with complete chromosomal complements is an alternative to single gene transfer.

VI. Transgenic Fish in Studies of Vertebrate Development

Powers (123) has comprehensively summarized the widespread advantages and uses of fish as a model system for biological research. But in the past two years the zebrafish in particular, originally developed by Streisinger and colleagues (139) who recognized the incredible superiority of a system that allowed the production of homozygous diploid vertebrates has been shown to be a powerful system for examining the genetics vertebrate development. Streisinger's legacy to developmental biology is just now being widely appreciated. Two recent, splendid reviews on the melding of classical genetics, transgenics, and developmental cell biology document in far more detail than space here permits the bright future of zebrafish for developmental genetics of vertebrates (128,138). Though lacking in characterized mutants, the zebrafish system offers the advantage of being capable of i) yielding many developmental mutatnts with markers in genetic loci involved in development and ii) allowing investigations of the genetic basis of early development using reverse genetics with genes isolated and cloned from other animals in a system wherein embryogenesis and organogenesis can be directly viewed as an ongoing process.

Transgenic techniques will play a major role in such studies in four ways. First, reporter genes linked to the enhancer/promoters of genes from other organisms (Fig. 2-2a) can be introduced into the embryo in order to determine the temporal and spatial expression patterns of the foreign gene regulatory sequences. Using the homeobox *Hox* genes (35), Westerfield (153) has used transient expression of Hox-promoters and mutants to drive *lacZ* gene transcription in mosaic transgenic zebrafish. A corollary experimental strategy is to place a reporter gene behind a minimal promoter and examine transgenic offspring for tissue-specific or developmental-specific gene expression. The specific activation of the transgene indicates integration in the neighborhood of a specific

enhancer element and is accordingly referred to as promoter or enhancer traps (104,116). Experiments of this kind will be invaluable for mapping enhancer/promoter function in vertebrates.

Second, inappropriate gene expression and its consequences can be determined using *gain-of-function* constructs (Fig. 2-1a,b,c) in which a normal fish gene or its homologue could be placed under the control of a different, possibly constitutive or strong, enhancer/promoter. For instance, the role of over expression of proto-oncogenes in carcinoma is not well understood. Placing a fish *c-myc* gene (150) or *Tu*-locus, melanoma-producing gene (156) behind a strong promoter or tissue specific promoter could lead to new understandings on the effects of over expression of oncogenes.

A third role of transgenic constructs is the converse of the above procedure, that is to orient the cDNA of the gene under investigation in the opposite orientation to make an antisense transcript that could inhibit normal expression (74). These constructs become *loss-of-function* mutants (Fig. 2-3a) even though they would leave the primary gene untouched. This procedure has been effective in mice. For example, b-glucuronidase gene activity, which is turned on 60-fold in the 4-cell to blastocyst stages of mouse preimplantation embryos, was inhibited by antisense RNA (7) and prothymosin antisense RNA oligomers inhibited myeloma cell division (130). Such a strategy has been mentioned as a potential weapon against viral infections (section V-3 above). A similar strategy, that requires more information about the sequence and secondary structure of the mRNA from the gene under investigation, is the use of catalytic RNA (Fig. 2-3b). Here a particular construction containing directing the synthesis of portions of the complement of the mRNA plus an RNA sequence capable of ribozyme digestion is placed either behind the normal promoter or behind a constitutive promoter (63). The result is an RNA that can seek out and destroy specific mRNAs and thereby achieve the same effect as antisense RNA. The advantage of catalytic RNAs that a large excess is not required since the RNA is catalytic rather than a competitive inhibitor. The disadvantage of catalytic RNA is the complexity of design of the killer transcript and the lack of precedent in its use.

The fourth role of transgenics, insertional mutagenesis and gene inactivation evident at specific periods of development, was pioneered in transgenic mice several years ago (47,84). The same procedures could be quite beneficial when applied to model fish systems since embryos are plentiful and irregular development can be directly visualized. Since the transgenic element is known, it provides a molecular probe for examining the genome of the damaged organism in order to determine the gene that was interrupted. Related to this procedure is the possibility of gene targeting (15,97).

Heretofore the procedure has been relatively inefficient and used only on embryonic stem cells in culture which are then injected into the developing animal (*e.g.*, 147). With the availability of large numbers of fertilized eggs, it may be possible to directly achieve gene targeting in developing zygotes by transgenic procedures.

In closing, one area of scientific inquiry that will greatly benefit from transgenic procedures is molecular endocrinology in fish (6). The needs of aquaculture for a better understanding of the endocrinology of reproduction, growth and environmental adaptation that occur in fish as well as the roles of steroid hormones in growth and development (31,167) may be met in part by research employing transgenic fish. The future and benefits of the research are bright and enticing.

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Exhibit 7

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Development of transgenic fish for ornamental and bioreactor by strong expression of fluorescent proteins in the skeletal muscle

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Abstract

In the present study, new applications of the transgenic technology in developing novel varieties of ornamental fish and bioreactor fish were explored in a model fish, the zebrafish (*Danio rerio*). Three "living color" fluorescent proteins, green fluorescent protein (GFP), yellow fluorescent protein (YFP), and red fluorescent protein (RFP or dsRed), were expressed under a strong muscle-specific *mylz2* promoter in stable lines of transgenic zebrafish. These transgenic zebrafish display vivid fluorescent colors (green, red, yellow, or orange) visible to unaided eyes under both daylight and ultraviolet light in the dark. The level of foreign protein expression is estimated between 3% and 17% of total muscle proteins, equivalent to 4.8–27.2 mg/g wet muscle tissue. Thus, the fish muscle may be explored as another useful bioreactor system for production of recombinant proteins. In spite of the high level of foreign protein expression, the expression of endogenous *mylz2* mRNAs was not negatively affected. Furthermore, compared to the wild-type fish, these fluorescent transgenic fish have no advantage in survival and reproduction.

Keywords: Zebrafish; Genetically modified organism; Green fluorescent protein; Red fluorescent protein; dsRed; Yellow fluorescent protein

The green fluorescent protein (GFP) originally isolated from the jellyfish (*Aequorea victoria*) is intrinsically fluorescent, allowing direct visualization without the need of substrate for chemical reaction. The cDNA for this protein has been cloned and modified by site-directed mutagenesis for different emission spectra and thus several artificial fluorescent color proteins become available, including yellow fluorescent protein (YFP), blue fluorescent protein (BFP), and cyan fluorescent protein (CFP) [1]. More recently, a new fluorescent protein cDNA encoding a red fluorescent protein (RFP or dsRed) has been cloned from the IndoPacific sea anemone relative (*Discosoma sp*) [2]. Due to the fact that these fluorescent proteins can be observed in live biological samples for labeling cells and subcellular organelles, these fluorescent proteins have been aptly termed "living colors" by Clontech.

The transgenic technology is widely used in biotechnology, from generation of genetically modified (GM) foods to production of pharmaceutical proteins. Inspired by the success of generation of super-mice using exogenously introduced *growth hormone* gene [3], this technology has been successfully used to develop fast-growing super-fish stocks for aquaculture. So far, fast-growing fish by transferring a growth hormone gene have been developed for several aquacultural species [4–11]; however, marketing of these transgenic food fish remains a controversial issue due to ecological and food safety concerns [12]. In this study, by taking advantages of "living color" fluorescent proteins, the feasibility of using the transgenic technology to develop novel varieties of ornamental fish was explored in zebrafish. Several stable lines of transgenic zebrafish expressing GFP, RFP, or YFP under a strong muscle-specific *mylz2* promoter were developed. These transgenic zebrafish display vivid fluorescent colors that are readily visible to unaided eyes. Meanwhile, we demonstrated that the fish muscle have a high capacity of expressing recombinant proteins without negative effect on the expression of the endogenous *mylz2* mRNA and thus may become another transgenic bioreactor system.

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Materials and methods

Production of transgenic zebrafish. Three transgenic DNA constructs used in the present studies: pMYLZ2-EGFP, pMYLZ2-RFP, and pMYLZ2-YFP were constructed by insertion of a 2-kb *mylz2* promoter into pEGFP-1, pEYFP-1, and pdsRed-1 (Clontech), respectively. *Mylz2*, short from *myosin light polypeptide 2*, is a fast skeletal muscle-specific gene [13] and was previously named *MLC2f* [14]. Transgenic zebrafish were generated by microinjection of plasmid DNAs in linearized form into embryos at 1- or 2-cell stage [15]. Transgenic lines were screened by direct observation of fluorescent protein expression in the offspring. Stable transgenic lines were established by standard breeding and confirmed by typical Mendelian inheritance ratios. These stable transgenic lines containing pMYLZ2-EGFP, pMYLZ2-RFP, and pMYLZ2-YFP are called *gfp*, *rfp*, and *yfp* transgenic fish, respectively, in this study.

Expression analysis. Estimation of the level of protein expression was carried out by SDS–polyacrylamide gel electrophoresis and quantified by the Gel-Pro Analyzer program (Media Cybernetics, USA). The content of total muscle protein was estimated by dissolving isolated muscle tissue to the SDS sample buffer (2% SDS; 7.5% glycerol; 5% 2-mercaptoethanol; and 80 mM Tris, pH 6.8) and protein concentration was determined by the method of Esen [16] using purified BSA (bovine serum albumin) (Merck) as standards. Transgenic and endogenous RNA expression was analyzed by Northern blot hybridization. Total RNAs were prepared from individual fish using Trizol reagent (Invitrogen) and autoradiograms were quantified by the Gel-Pro Analyzer program.

Survival rates. For comparison of survival rates (Table 1), eggs were collected from crosses between two hemizygous *gfp* and *rfp* transgenic zebrafish. About 25% of individuals with each of the four genotypes were obtained: wild type, *gfp*, *rfp*, and *gfp/rfp*. The genotypes/phenotypes were determined in fry of 3–4 day postfertilization when both RFP and GFP expression can be easily detected. These offspring from the same pair of parents were cultured in the same tank and counted again at the adult stage.

Reproduction success. Reproduction success was measured by counting the numbers of embryos produced from a pool of equal number of the following four types of spawning fish of similar size: wild-type males, wild-type females, *gfp* transgenic males, and *gfp* transgenic females. Each fish selected was pre-tested individually to ensure that it spawned actively. Eggs were collected for 7–8 consecutive days and the embryos were examined under a fluorescent microscope. The penetrance of phenotype is always 100%, as confirmed by PCR analysis.

Results

Expression of vivid fluorescent colors in stable lines of transgenic zebrafish

To generate fluorescent transgenic zebrafish, the three transgenic DNA constructs, pMYLZ2-EGFP, pMYLZ2-RFP, and pMYLZ2-YFP, were injected separately into zebrafish embryos at 1–2 cell stage. Stable transgenic lines were obtained from all of the three recombinant DNA constructs. In all transgenic lines, fluorescent proteins were highly expressed in the skeletal muscle, in a pattern faithfully mimicking the endogenous *mylz2* expression [15,17]. Due to the extremely high level of expression in the skeletal muscle and the large mass of muscle tissue, green, yellow, and red fluorescent colors were readily visualized even under normal day light (Figs. 1A and C). Under an ultraviolet light in the dark, these transgenic zebrafish displayed vivid fluorescent colors to unaided eyes (Figs. 1B and D). The transgenic fluorescent colors became visible at about four weeks postfertilization and intensified in the following weeks. Since the generation of the first *gfp* transgenic line in 1999, the *gfp* line is currently at its eighth generation, the *yfp* line at its fourth, and the *rfp* line at its sixth. In all these generations, the same intensity of fluorescence was observed in a total of a few thousand offspring produced in our laboratory, suggesting that the transgenes and fluorescent phenotypes have been stably inherited.

To investigate the possibility of creating more "rainbow" colors, double transgenic fish were obtained by crossing a *gfp* fish with an *rfp* fish. The double transgenic fish (*gfp/rfp*) displayed an orange color both under daylight and ultraviolet light (Fig. 1). To test the feasibility of generating more intermediate colors, muscle extracts from *gfp* fish and *rfp* fish were mixed at different proportions; a series of intermediate colors were obtained (Fig. 1E). Based on our previous observations by transient and stable transgenic assays, the level of GFP expression was related to the length of *mylz2* promoter and a weaker expression was obtained by a shorter promoter [17]. Thus, the level and spectrum of transgenic expression under the *mylz2* promoter could be further manipulated for more transgenic colors.

High level of muscle expression of foreign proteins in transgenic fish

The levels of GFP and RFP expression in *gfp* and *rfp* transgenic zebrafish were estimated by SDS–polyacrylamide gel electrophoresis. Total muscle proteins were extracted and analyzed. Both GFP and RFP were easily

Table 1
Survival rates of offspring of different genotypes between crosses of *gfp* and *rfp* transgenic zebrafish

Genotypes	*gfp*	*rfp*	*gfp/rfp*	wild type
Batch 1	53.1% (17/32)	64.0% (16/25)	67.9% (19/28)	66.7% (16/24)
Batch 2	68.9% (20/29)	100% (26/26)	86.9% (20/23)	86.2% (25/29)
Batch 3	43.5% (20/46)	57.4% (27/47)	54.8% (23/42)	64.3% (27/42)
Average	55.2%	73.8%	69.9%	72.4%

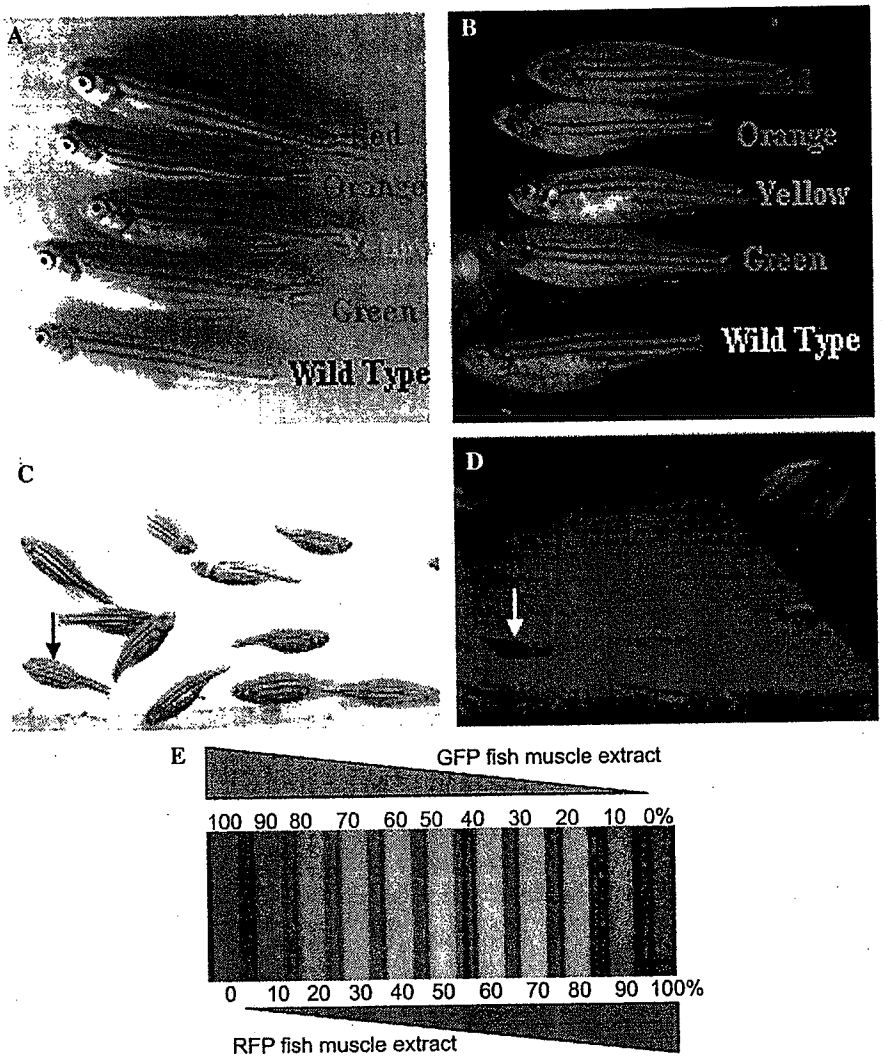

Fig. 1. Fluorescent transgenic zebrafish. (A,B) Fluorescent transgenic zebrafish in a rainbow array (top to bottom) under daylight (A) and 385 nm ultraviolet light (B). Red, *rfp* fish; orange, *rfp*/*gfp* fish; yellow, *yfp* fish; green, *gfp* fish; and wild-type fish. These fish were anesthetized in 0.1% phenoxyethanol and photographed with a digital camera. (C,D) Living color fluorescent transgenic zebrafish in swimming under daylight (C) and 385 nm ultraviolet light (D). Arrows in (C) and (D) indicate wild-type fish. (E) Intermediate colors by blending of GFP and RFP. Muscle extracts in phosphate buffered saline were prepared from *gfp* and *rfp* transgenic fish and mixed in different ratios. These mixtures were then loaded into capillary tubes and photographed under a microscope using a GFP plus filter (Leica). The proportions of GFP and RFP muscle extracts used are indicated at both the top and bottom of each tube.

identified as they appeared in the transgenic samples as additional protein bands with expected sizes (Fig. 2A). As analyzed by the Gel-Pro Analyzer program, we estimated that GFP and RFP were expressed at the level of ~3% and ~5% of total muscle proteins in *gfp* and *rfp* transgenic zebrafish (hemizygotes), respectively (Fig. 2A). In *gfp*/*rfp* double transgenic zebrafish, both levels remained the same. In homozygotes, the level of expression appeared to be doubled (Fig. 2B). Using purified bovine serum albumin as standards, we estimated that a homozygous *rfp* fish synthesizes the recombinant protein as high as 17% of total muscle proteins. We also measured the amount of protein per gram of wet muscle tissue to be about 160 mg. Thus, the transgenic fish muscle system could generate 4.8–27.2 mg of single recombinant protein in one gram of wet muscle tissue.

To examine whether endogenous *mylz2* mRNA expression is affected by such high level of transgene expression, Northern blot hybridization was carried out. As shown in Fig. 3, the endogenous *mylz2* mRNA expression was not inhibited in either male or female *gfp* transgenic zebrafish. Identical results were also obtained from the *rfp* transgenic line (data not shown). Real-time PCR analysis indicates that the *gfp* transgenic line contained only about two copies of *mylz2-gfp* transgene in a haploid genome, but the expression of *gfp* mRNA in the hemizygous transgenic fish is at least 10-fold higher than that of endogenous *mylz2* mRNA in the control fish (Fig. 3C). Therefore, these experiments demon-

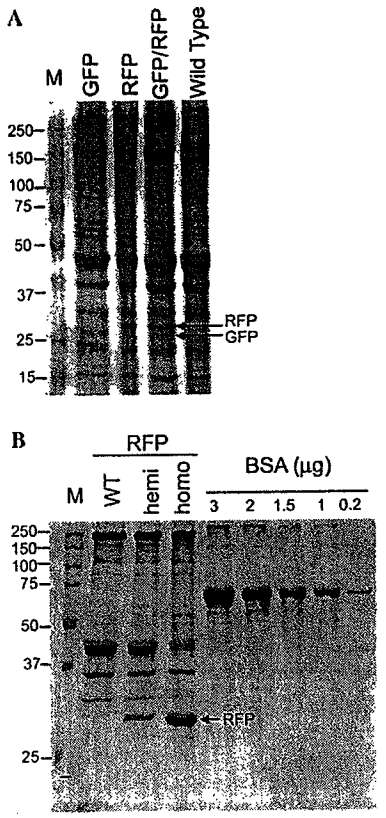

Fig. 2. Expression of GFP and RFP in transgenic zebrafish. Total muscle proteins were separated by SDS gel electrophoresis and stained with Coomassie Blue-G-250. Prestained protein standards (lane M) were used and molecular weights (Bio-Rad) are shown on the left of each panel. (A) Expression of GFP and RFP in *gfp*, *rfp*, and *gfp/rfp* transgenic zebrafish. GFP and RFP are indicated by arrows. (B) Quantification of the level of RFP expression in *rfp* transgenic zebrafish. 10 μg of protein each from wild type (WT), hemizygous (hemi), and homozygous (homo) *rfp* transgenic zebrafish was loaded on each lane. Different amounts of purified BSA, as indicated at the top of each lane, were loaded for a standard curve. The gels were analyzed by Gel-Pro Analyzer program. The amount of recombinant RFP was estimated at ~0.5 μg for the hemizygous sample and 1.7 μg for the homozygous sample.

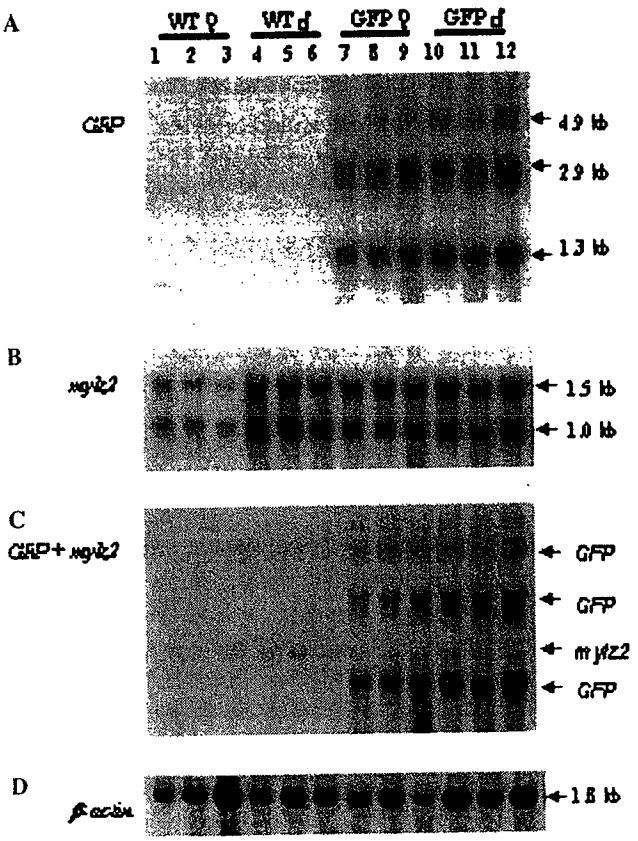

Fig. 3. No reduction of endogenous *mylz2* mRNA expression by high level of transgenic expression. Total RNAs were prepared from whole fish of three each of wild-type female, wild-type male, *gfp* transgenic female, and *gfp* transgenic male. Northern blot analysis was performed on the same RNA blot that was hybridized with *gfp* (A), *mylz2* (B), *gfp*/*mylz2* mix (C), and β-*actin* (D) probes, respectively, after the blot was repeatedly striped. Two *mylz2* transcripts were detected due to differential polyadenylation sites (B). To compare the relative expression levels of *mylz2* and *gfp* mRNAs, the same length of probes for *mylz2* (657 bp) and *gfp* (657 bp) was generated by PCR and labeled to the same specific activity in the same reaction tube with equal quantity of DNA templates. The autoradiogram is shown in (C) and the relative intensities should represent the relative levels of *mylz2* and *gfp* mRNA expression. The *mylz2* probe used in (C) was designed in the 3′UTR and only the 1.5-kb transcript was hybridized. The level of the three major *gfp* mRNAs is at least 10-fold higher than that of endogenous *mylz2* mRNA as estimated by the Gel-Pro Analyzer. The phenotype and sex of each fish are indicated at the top of each lane, the probes used are indicated on the left, and the size and names of the transcripts on the right.

strated that high level of muscle expression of a foreign gene can be achieved without any compromise of endogenous gene expression. Consistent with this, we did not notice any abnormal swimming behaviour and morphology associated with the transgenic fish.

Lack of evidence for enhanced fitness in fluorescent transgenic fish

Previously, Muir and Howard [18] proposed a Trojan gene hypothesis on large-sized *growth hormone* transgenic fish based on a mathematic model. According to this hypothesis, if the larger *growth hormone* transgenic fish have a lower viability and a favored mating behavior, accidental release of such transgenic fish will cause the extinction of the wild-type population in about 40 generations. To investigate whether the transgenesis will affect survival and reproduction of our fluorescent transgenic fish, both survival rates and the number of eggs produced were compared between transgenic and wild-type fish. The survival rates of transgenic fish were assessed from the offspring of crosses between a hemizygous *gfp* fish and a hemizygous *rfp* fish under our laboratory condition. These crosses produced four genotypes/phenotypes at a ratio of 1:1:1:1, wild type, *gfp* (green), *rfp* (red), and *gfp/rfp* (orange). As shown in Table 1, with the exception that *gfp* fish appeared to have a lower survival rate, there was no apparent

difference in survival rates among the other three phenotypes. To evaluate the reproduction success, a population of equal numbers of wild type and hemizygous *gfp* transgenic fish was maintained for breeding for a week (see Materials and methods). The expected ratio (43.75%) of transgenic offspring based on Mendelian genetics was observed (43.60%, 1158 out of 2656). Similar result was obtained from a second experiment (40.60%, 2121 out of 5224). Thus, there is no indication that the fluorescent transgenic fish would have any reproductive advantage. Overall, these experiments provided no evidence that the acquirement of fluorescent colors could bring about a better fitness for these transgenic fish.

Discussion

In the present study, the zebrafish was explored as a model to demonstrate the feasibility of using transgenic technology to generate new varieties of ornamental fish. It is obvious that this technology can be applied to other more exotic ornamental fish species, such as goldfish, Japanese koi (carp), etc. Using the same zebrafish promoter construct, pMYLZ2-EGFP, we recently generated a transgenic medaka line that also displayed visible green fluorescent color like *gfp* transgenic zebrafish (Z. Zeng and Z. Gong, unpublished). In the ornamental fish industry, the color pattern is also important. By using different tissue-specific promoters, these color proteins could be targeted into different tissues or body parts. Particularly, different variety of color patterns can be achieved from different combinations of tissue-specific promoters and color proteins. As only a limited number of new varieties of ornamental fish can be produced currently by classical breeding, the transgenic approach will be a new avenue for rapid production of novel ornamental fish. Furthermore, once the fluorescent transgenic fish is available for one strain, it is easy to transfer the transgene to other useful strains within the same species by standard genetic breeding for more varieties of fluorescent transgenic fish. Currently more and more fluorescent protein genes have been cloned from reef coral species [19] and the choice of original colors widens. New color can be further blended genetically as shown in this study (Fig. 1E). Thus, the application of transgenic technology in ornamental fish is promising.

The success of generating transgenic zebrafish with visible fluorescent colors is due to an unusually high level of expression of these fluorescent proteins in the muscle tissues. The promoter we used in the present study is derived from the muscle-specific *mylz2* gene. Previously, by analysis of transcript profile using an EST approach, we have shown that *mylz2* clone is the most abundant one in both zebrafish embryos and adults [20]. Thus, the *mylz2* promoter is likely the strongest muscle-specific promoter. So far, *gfp* transgenic zebrafish have been produced using many different tissue-specific promoters (e.g. [21–28]), but none of these transgenic lines display fluorescent color visible to unaided eyes. Thus, one key to success in the generation of colorful transgenic ornamental fish is in the strength of the promoter. Another factor is the selection of tissue; the muscle constitutes majority of the body and thus synthesizes more and visible color proteins. In contrast, transgenic GFP expression in only a single layer of skin cells cannot be visualized without using a fluorescent microscope [27].

At present, marketing of transgenic food fish is facing two major challenges, environmental concerns and food safety [12]. In contrast, the ornamental fish aquaculture is generally practiced in small and well-contained environments, and most ornamental fish species cannot survive in the wild after a long period of domestic selection and breeding. Our current study indicated that the fluorescent transgenic fish has no advantages in survival and reproduction. A preliminary study on mate choice between wild type and fluorescent transgenic zebrafish, based on a dichotomous choice test on guppies [29], also indicated the lack of mating advantage of transgenic zebrafish (W.K. Seah, D. Li, M. Chen, and Z. Gong, unpublished). Thus, the culturing of GM ornamental fish should be less concerned with regard to environmental and ecological issues. Moreover, there is no food safety issue for ornamental fish. Therefore, marketing of transgenic ornamental fish is a more viable concept.

Another important finding of this study is the extremely high level of muscle expression of foreign proteins in the transgenic fish. We have estimated that the fish muscle has a capacity of producing up to 27 mg of foreign protein per gram of wet tissue. This is comparable to or greater than the level of recombinant protein expression (~10 mg/ml) in the popular mammary gland system in transgenic farm animals [30] and is significantly higher than that (~1 μg/ml) in the egg white of transgenic hen [31]. Hence, the fish muscle could be developed to another transgenic bioreactor system. The advantages of the transgenic fish bioreactor include the speed of generation of transgenic fish, low cost, and lack of risk of transferring mammalian viruses and prions, etc. Furthermore, the fish muscle is edible and raw meat may be directly consumed for better preservation of bioactivity; thus, nutritional and pharmaceutical/therapeutical supplements could be expressed in the fish muscle system. To create a more successful fish bioreactor system, fast-growing and large size farm fish such as carp, tilapia, catfish, salmon, and rainbow trout may be used. For all these species, the transgenic technology is well developed [3–11].

Acknowledgments

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Exhibit 8

#120 Date 5/30/03

CERTIFICATE OF MAILING
37 C.F.R. 1.8

I hereby certify that this correspondence is being deposited with the U.S. Postal Service with sufficient postage as First Class Mail in an envelope addressed to: Commissioner for Patents, P. O. Box 1450, Alexandria, VA 22313-1450, on the date below:

May 9, 2003
Date

David L. Parker

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Gong et al.

Serial No.: 09/913,898

Filed: October 3, 2001

For: CHIMERIC GENE CONSTRUCTS FOR GENERATION OF FLUORESCENT TRANSGENIC ORNAMENTAL FISH

Group Art Unit: 1632

Examiner: Joseph T. Woitach

Atty. Dkt. No.: GLOF:007US

I. AMENDMENT; II. RESPONSE TO RESTRICTION REQUIREMENT DATED MARCH 13, 2003; AND III. REQUEST FOR EXTENSION OF TIME

Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

Commissioner:

This paper is submitted in response to the Restriction Requirement dated March 13, 2003 for which the date for response was April 13, 2003.

A request for a one-month extension of time to respond is included herewith along with the required fee. This extension will bring the due date to May 13, 2003, which is within the six-month statutory period. Should such request or fee be deficient or absent, consider this paragraph such a request and authorization to withdraw the appropriate fee under 37 C.F.R. §§ 1.16 to 1.21 from Fulbright & Jaworski L.L.P. Account No.: 50-1212/GLOF:007US.

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I. AMENDMENT

Please cancel pending claims 1-18 without prejudice or disclaimer, and introduce the following new claims, claims 19-59:

19. A method of providing transgenic fish to the ornamental fish market, comprising the steps of:

(a) obtaining an ornamental transgenic fish comprising one or more chimeric fluorescence genes positioned under the control of a promoter, wherein the transgenic fish expresses one or more fluorescent proteins encoded by the one or more fluorescence genes at a level sufficient such that said fish fluoresces upon exposure to one or more of a blue light, ultraviolet light or sunlight; and

(b) distributing said fish to the ornamental fish market.

20. The method of claim 19, further comprising displaying said transgenic fish under a blue or ultraviolet light.

21. The method of claim 20, wherein the transgenic fish are displayed under an ultraviolet light that emits light at a wavelength selected to be optimal for the fluorescent protein or proteins.

22. The method of claim 21, wherein the transgenic fish comprise a GFP and are displayed under an ultraviolet light that emits light at 365 nm.

23. The method of claim 21, wherein the transgenic fish comprise a GFP and are displayed under an ultraviolet light that emits light at 395 nm.

24. The method of claim 21, wherein the transgenic fish comprise a GFP and are displayed under a blue light that emits light at 488 nm.

25. The method of claim 19, wherein the transgenic fish express a GFP.

26. The method of claim 26, wherein the transgenic fish express an EGFP.

27. The method of claim 19, wherein the transgenic fish express a BFP.

28. The method of claim 27, wherein the transgenic fish express an EBFP.

29. The method of claim 19, wherein the transgenic fish express a YFP.

30. The method of claim 29, wherein the transgenic fish express an EYFP.

31. The method of claim 19, wherein the transgenic fish express a CFP

32. The method of claim 31, wherein the transgenic fish express an ECFP.

33. The method of claim 19, wherein the transgenic fish expresses more than one color of fluorescent protein.

34. The method of claim 19, wherein the promoter is a tissue specific promoter.

35. The method of claim 34, where the promoter is a skin specific promoter.

36. The method of claim 35, wherein the promoter is a zebrafish cytokeratin gene promoter.

37. The method of claim 34, wherein the promoter is a muscle specific promoter.

38. The method of claim 37, wherein the promoter is a zebrafish muscle creatine kinase gene promoter.

39. The method of claim 37, wherein the promoter is a zebrafish myosin light chain 2 gene promoter.

40. The method of claim 34, wherein the promoter is an eye specific promoter.

41. The method of claim 34, wherein the promoter is a bone specific promoter.

42. The method of claim 19, wherein the promoter is a ubiquitously expressing promoter.

43. The method of claim 42, wherein the promoter is a zebrafish acidic ribosomal protein gene promoter.

44. The method of claim 19, wherein the promoter is an inducible promoter.

45. The method of claim 44, wherein the inducible promoter is a hormone inducible promoter.

46. The method of claim 44, wherein the inducible promoter is a heavy metal inducible promoter.

47. The method of claim 34, wherein the transgenic fish expresses more than one fluorescent protein color.

48. The method of claim 47, wherein the more than one fluorescent protein is expressed in the same tissue, to effect a new fluorescent color.

49. The method of claim 48, where the transgenic fish expresses a GFP and a BFP.

50. The method of claim 47, wherein the more than one fluorescent proteins are separately expressed in different tissues.

51. The method of claim 50, wherein the transgenic fish expresses a GFP under the control of an eye specific promoter.

52. The method of claim 50, wherein the transgenic fish expresses a BFP under the control of a skin specific promoter.

53. The method of claim 50, wherein the transgenic fish expresses a YFP under the control of a muscle specific promoter.

54. The method of claim 19, wherein the transgenic fish is a stable transgenic fish line obtained by a method comprising the steps of:

(a) obtained an ornamental transgenic fish comprising one or more chimeric fluorescence genes positioned under the control of a promoter, wherein the transgenic fish expresses one or more fluorescent proteins encoded by the one or more fluorescence genes at a level sufficient such that said fish fluoresces upon exposure to one or more of a blue light, ultraviolet light or sunlight; and

(b) breeding the ornamental transgenic fish with a second fish to obtain offspring; and

(c) selecting from said offspring a stable transgenic line that expresses one or more fluorescent proteins.

55. The method of claim 54, wherein the second fish is a wild type fish.

56. The method of claim 54, wherein the second fish is a second transgenic fish.

57. The method of claim 19 or 54, wherein the ornamental transgenic fish is a transgenic zebrafish, medaka, goldfish or carp.

58. The method of claim 54, wherein the second fish is a zebrafish, medaka, goldfish or carp.

59. The method of claim 19 or 54, wherein the ornamental transgenic fish is a transgenic koi, loach, tilapia, glassfish, catfish, angel fish, discus, eel, tetra, goby, gourami, guppy, Xiphophorus, hatchet fish, Molly fish, or pangasius.

The pending claims are attached hereto as Exhibit A.

II. RESPONSE TO RESTRICTION REQUIREMENT

In response to the restriction requirement which the Examiner imposed, Applicants have elected to proceed with a new set of claims, claims 19-59, directed to what is in essence a "method of doing business." This particular method of doing business is the idea of providing certain types of transgenic fish, particularly fluorescent transgenic fish, to the ornamental fish market. For this reason, it is not believed that the current claims fit into any of the restriction groups identified by the Examiner with respect to the previous claims. However, in that there is only a single independent claim, it is appropriate for these claims to co-exist in a single application, if the independent claim is found allowable. See 37 C.F.R. §1.141.

Support for the newly added claims is as follows:

The disclosure of providing fluorescent transgenic fish to the ornamental fish market found in claim 19 is supported by the specification (original PCT specification) at, for example, page 3, lines 22-30, and in Example IV, pages 21-23.

The disclosure of a fish that fluoresces upon exposure to one or more of a blue light, ultraviolet light or sunlight can be found throughout the specification, for example, figures 8-11 and page 20, lines 13-18.

The disclosure of using a light that is optimal for the particular fluorescence (claim 21), as well as the disclosure of GFP (green fluorescent protein) display and emission at 365 nm, 395 nm and 488 nm (claims 22-24) can be found, for example, in Figures 9-10; Figure 11 (showing green fluorescent fish at 365 nm and discussing fluorescence at 488 nm, see page 6, lines 21-24); page 10, lines 19-22; and at page 2, lines 31-31 (395 nm).

The disclosure of the various fluorescence protein genes set forth in claims 26-32 can be found at page 9, lines 22-34.

The disclosure of expressing more than one color (claim 33 and 47) as well as the disclosure of combining two colors in the same tissue to make a third color (48-49) or to make different colors in different tissues (claim 50) can be found in the specification, for example, at page 21, line 29, to page 22, line 4.

Disclosure of the various promoters set forth in claims 34-46 can be found throughout the specification, for example, page 3, line 22 though page 4, line 18; Example II; Example IV, page 21, line 29 to page 22, line 4; page 10, lines 21-22.

Support for fish species other than zebrafish is found at page 22, line 28, though page 23, line 13.

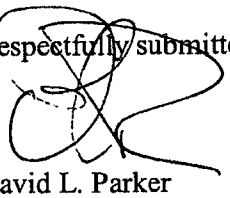
III. REQUEST FOR EXTENSION OF TIME

Pursuant to 37 C.F.R. § 1.136(a), Applicants request an extension of time of one month to and including May 13, 2003 in which to respond to the Office Action dated March 13, 2003. Pursuant to 37 C.F.R. § 1.17, the extension fee is $55.00. A check is enclosed. Should any additional fees under 37 C.F.R. §§ 1.16 to 1.21 be required for any reason relating to the

enclosed materials, or should an overpayment be included herein, the Commissioner is authorized to deduct or credit said fees from or to Fulbright & Jaworski Deposit Account No. 50-1212/GLOF:007US.

The Examiner is invited to contact the undersigned attorney at (512) 536-3055 with any questions, comments or suggestions relating to the referenced patent application.

Respectfully submitted,



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Date: May 9, 2003

Exhibit 9

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ART UNIT	**PAPER NUMBER**
1632	

DATE MAILED: 12/18/2003

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No.	Applicant(s)	
	09/913,898	GONG ET AL.	
	Examiner	Art Unit	
	Joseph T. Woitach	1632	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 1 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133).
- Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *02 September 2003*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *19-62* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☐ Claim(s) _____ is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☒ Claim(s) *19-62* are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. §§ 119 and 120

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

13)☐ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. § 119(e) (to a provisional application) since a specific reference was included in the first sentence of the specification or in an Application Data Sheet. 37 CFR 1.78.

a) ☐ The translation of the foreign language provisional application has been received.

14)☐ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. §§ 120 and/or 121 since a specific reference was included in the first sentence of the specification or in an Application Data Sheet. 37 CFR 1.78.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO-1449) Paper No(s) _____.
4) ☐ Interview Summary (PTO-413) Paper No(s). _____.
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: .

DETAILED ACTION

This application is a 371 national stage filing of PCT/SG99/00079, filed July 16, 1999 which claims benefit to foreign application 9900811-2, filed February 18, 1999 in Singapore.

Examiner's comment

As indicated in the office action mailed Applicants amendment filed May 12, 2003, paper number 12, was been received and entered. Claims 1-18 were canceled and claims 19-59 were entered. Claims 19-59 were pending.

Presently, Applicant's amendment filed September 2, 2003, has been received and entered. The numbering of claims is not in accordance with 37 CFR 1.126 which requires the original numbering of the claims to be preserved throughout the prosecution. When claims are canceled, the remaining claims must not be renumbered. When new claims are presented, they must be numbered consecutively beginning with the number next following the highest numbered claims previously presented (whether entered or not). Misnumbered claims 19-21 have been renumbered 60-62.

Claims 19-62 are pending. In addition, the amendments proposed for claims 1-18 have not been entered because these claims have been canceled. Further, it is noted that some of the proposed claim amendments include dependency on the proposed canceled claims (for example claim 7 would depend on claims 1, 2, 3, or 4). Applicants election of Group VII, claims 7-13 and

16, is noted. However, these claims do not exist and newly added claims directed to the subject matter are not pending.

Appropriate correction is required. It is suggested that non-elected claims 19-62 be canceled, and that claims directed to Applicants' elected invention be added. It is noted that any new claim set should start number at claim 63.

Election/Restriction

Applicants' response to the restriction requirement is noted. However, as discussed above, the amendment filed on May 12, 2003, paper number 12, canceling all claims drawn to the inventions set forth in the restriction requirement and presenting only claims drawn to a new invention is **non-responsive** (MPEP § 821.03). As acknowledged previously by Applicants, the claims 19-59 are not readable on the elected invention. Claims 60-62 are dependent on canceled claim 10 and thus, not drawn to the elected invention.

Since the above-mentioned amendment appears to be a bona fide attempt to reply, applicant is given a TIME PERIOD of ONE (1) MONTH or THIRTY (30) DAYS, whichever is longer, from the mailing date of this notice within which to supply the omission or correction in order to avoid abandonment. EXTENSIONS OF THIS TIME PERIOD UNDER 37 CFR 1.136(a) ARE AVAILABLE.

The original restriction requirement is re-iterated below for Applicants' convenience.

Restriction is required under 35 U.S.C. 121 and 372.

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1.

In accordance with 37 CFR 1.499, applicant is required, in reply to this action, to elect a single invention to which the claims must be restricted.

I. Claims 1, 5, 6, 14, 15, drawn to a zebrafish cytokeratin gene promoter, classified in class 536, subclass 24.1.

II. Claims 2, 5, 6, 14, 15, drawn to a zebrafish creatine kinase gene promoter, classified in class 536, subclass 24.1.

III. Claims 3, 5, 6, 14, 15, drawn to a zebrafish fast muscle isoform of myosin light chain 2 gene promoter, classified in class 536, subclass 24.1.

IV. Claims 4, 5, 6, 14, 15, drawn to a zebrafish ribosomal protein P0 gene promoter, classified in class 536, subclass 24.1.

V. Claims 7-13, 16, drawn a transgenic fish comprising a zebrafish cytokeratin gene promoter, classified in class 800, subclass 20.

VI. Claims 7-13, 16, drawn a transgenic fish comprising a zebrafish creatine kinase gene promoter, classified in class 200, subclass 20.

VII. Claims 7-13, 16, drawn a transgenic fish comprising a zebrafish fast muscle isoform of myosin light chain 2 gene promoter, classified in class 800, subclass 20.

VIII. Claims 7-13, 16, drawn a transgenic fish comprising a zebrafish ribosomal protein P0 gene promoter, classified in class 800, subclass 20.

IX. Claim 17, drawn a transgenic fish comprising a zebrafish ribosomal protein P0 gene promoter, classified in class 800, subclass 20.

X. Claim 18, drawn a transgenic fish comprising a zebrafish ribosomal protein P0 gene promoter, classified in class 800, subclass 20.

Claims 5, 6, 14, 15 are generic to groups I-IV and will be examined to the extent they encompass the elected invention. Claims 7-13, 16 are generic to groups V-VIII and will be examined to the extent they encompass the elected invention.

The inventions listed as Groups I-X do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

A) The invention has no special technical feature that defined the contribution over the prior art, or

B) Unity of invention between different categories of inventions will only be found to exist if specific combinations of inventions are present. Those combinations include:

1) A product and a special process of manufacture of said product.

2) A product and a process of use of said product.

3) A product, a special process of manufacture of said product, and a process of use of said product.

4) A process and an apparatus specially designed to carry out said process.

5) A product, a special process of manufacture of said product, and an apparatus specially designed to carry out said process.

The allowed combinations do not include multiple products, multiple methods of using said products, and methods of making multiple products as claimed in the instant application, see MPEP § 1850. In the instant case, Applicant's claims encompass multiple inventions and do not have a special technical feature which link the inventions one to the other, and lack unity of invention.

Specifically, the inventions are distinct, each from the other because of the following reasons:

Inventions I-IV are unrelated. Inventions are unrelated if it can be shown that they are not disclosed as capable of use together and they have different modes of operation, different functions, or different effects (MPEP § 806.04, MPEP § 808.01). In the instant case the different polynucleotide sequences represent unique and different promoter sequences with different inherent properties as demonstrated by a particular expression pattern in a cell.

Inventions V-VIII are unrelated. Inventions are unrelated if it can be shown that they are not disclosed as capable of use together and they have different modes of operation, different

functions, or different effects (MPEP § 806.04, MPEP § 808.01). In the instant case each of the transgenic fish comprise a different promoter which results in a material different genome in the transgenic fish. Additional, the inherent activity of each of the promoters if expressed may provide a unique phenotype to each of the transgenic fish of each of the inventions.

Inventions I-IV and V-VIII, respectively, are related as product and process of use. The inventions can be shown to be distinct if either or both of the following can be shown: (1) the process for using the product as claimed can be practiced with another materially different product or (2) the product as claimed can be used in a materially different process of using that product (MPEP § 806.05(h)). In the instant case the promoters can be used for expression studies in vitro, as probes for detecting the presence of the respective gene, or as a target sequence for an anti-sense oligonucleotide.

Inventions IX and X are unrelated. Inventions are unrelated if it can be shown that they are not disclosed as capable of use together and they have different modes of operation, different functions, or different effects (MPEP § 806.04, MPEP § 808.01). In the instant case the methods require a transgenic fish comprising different promoters. The genome of the transgenic fish in each case are different and unique thus the methods require different starting materials. Further, the methods require the administration and analysis of two different classes of compounds; steroid-like compounds and heavy metals.

Inventions V-VIII and IX-X are unrelated. Inventions are unrelated if it can be shown that they are not disclosed as capable of use together and they have different modes of operation,

different functions, or different effects (MPEP § 806.04, MPEP § 808.01). In the instant case the methods require a transgenic fish comprising promoters which are different from those specifically set forth in the inventions of groups V-VIII.

Because these inventions are distinct for the reasons given above and have acquired a separate status in the art because of their recognized divergent subject matter, restriction for examination purposes as indicated is proper. Further, because these inventions are distinct for the reasons given above and the search required for each on of Group I-X is not required or coextensive with each other, restriction for examination purposes as indicated is proper.

Applicant is advised that the reply to this requirement to be complete must include an election of the invention to be examined even though the requirement be traversed (37 CFR 1.143).

Applicant is reminded that upon the cancellation of claims to a non-elected invention, the inventorship must be amended in compliance with 37 CFR 1.48(b) if one or more of the currently named inventors is no longer an inventor of at least one claim remaining in the application. Any amendment of inventorship must be accompanied by a request under 37 CFR 1.48(b) and by the fee required under 37 CFR 1.17(i).

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Joseph Woitach whose telephone number is (703)305-3732.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Deborah Reynolds, can be reached at (703)305-4051.

Any inquiry of a general nature or relating to the status of this application should be directed to the Group analyst Dianiece Jacobs whose telephone number is (703) 308-2141.

Joseph T. Woitach

Joe Woitach
AU1632

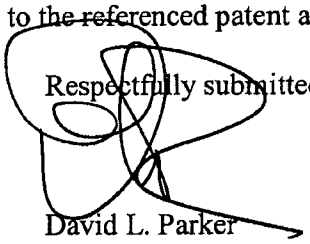
REMARKS; RESPONSE TO RESTRICTION

Claims 1-6, 12, 14-15 and 17-18 have been cancelled without prejudice, claim 9 was amended and new claims 19-21 added. New claims 19-21 correspond generally to original claims 8, 9 and 11, with the exception that they depend from claim 10 rather than claim 7. Additionally, claim 19 is specifically drawn to ornamental fish. Support for the ornamental fish *per se* can be found in the specification in Example IV.

In response to the restriction requirement which the Examiner imposed, Applicants elect, without traverse, to prosecute claims 7-13 and 16, *i.e.*, the Group VII claims. We understand that the claims will initially be examined to the extent they read on the zebrafish myosin light chain promoter and that, if allowable, the claim will be examined generically.

The Examiner is invited to contact the undersigned attorney at (512) 536-3055 with any questions, comments or suggestions relating to the referenced patent application.

Respectfully submitted,



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Date: August 27, 2003

AMENDMENTS

Listing of Claims

The following listing of claims replaces all previous listings or versions thereof:

1. (Currently amended) The method of claim 43, further defined as ~~A method of providing transgenic fish to the ornamental fish market,~~ comprising the steps of:

(a) obtaining a transgenic fish line comprising one or more chimeric ~~fluorescence~~ genes that are positioned under the control of a promoter that drives the expression of a fluorescent protein in muscles of said fish, said promoter being a muscle specific promoter, such that said transgenic fish expresses fluorescent protein encoded by the gene in skeletal muscle at a level sufficient such that said transgenic fish fluoresces upon exposure to sunlight, wherein said transgenic fish are the offspring of an embryo line visually exhibiting expression of the fluorescent protein in essentially all muscle fibers in their trunk and further wherein transgenic founders of said line fluoresce upon exposure to sunlight~~, wherein the transgenic fish expresses one or more fluorescent proteins selected from the group of fluorescent proteins consisting of a blue fluorescent protein, a yellow fluorescent protein and a cyan fluorescent protein, encoded by the one or more fluorescence genes~~; and

(b) distributing fish of said ~~fish~~ line to the ornamental fish market.

2. (Currently amended) The method of claim 1 or claim 43, further comprising displaying said transgenic fish under a blue or ultraviolet light.

3. (Original) The method of claim 2, wherein the transgenic fish are displayed under an ultraviolet light that emits light at a wavelength selected to be optimal for the fluorescent protein or proteins.

4. – 8. (Cancelled)

9. (Currently amended) The method of claim 1 or claim 43, wherein the transgenic fish express a BFP.

10. (Original) The method of claim 9, wherein the transgenic fish express an EBFP.

11. (Currently amended) The method of claim 1 or claim 43, wherein the transgenic fish express a YFP.

12. (Original) The method of claim 11, wherein the transgenic fish express an EYFP.

13. (Currently amended) The method of claim 1 or claim 43, wherein the transgenic fish express a CFP

14. (Original) The method of claim 13, wherein the transgenic fish express an ECFP.

15. (Currently amended) The method of claim 1 or claim 43, ~~A method of providing transgenic fish to the ornamental fish market, comprising the steps of:~~

~~(a) obtaining a transgenic fish comprising fluorescence genes positioned under the control of a promoter,~~ wherein the transgenic fish expresses more than one color of fluorescent protein encoded by the ~~fluorescence~~ gene or genes~~; and~~

~~(b) distributing said fish to the ornamental fish market~~.

16. – 19. (Cancelled)

20. (Currently amended) The method of claim 1 or claim 43~~claim 19~~, wherein the promoter is a zebrafish muscle creatine kinase gene promoter.

21. (Currently amended) The method of ~~claim 19~~claim 1 or claim 43, wherein the promoter is a zebrafish myosin light chain 2 gene promoter.

22. – 23. (Cancelled)

24. (Currently amended) The method of claim 1 or claim 43 ~~or 15~~, ~~wherein the promoter is~~ wherein one or more of said chimeric genes further comprises a ubiquitously expressing promoter.

25. – 29. (Cancelled)

30. (Previously presented) The method of claim 15, wherein the more than one fluorescent protein is expressed in the same tissue, to effect a new fluorescent color.

31. (Original) The method of claim 30, where the transgenic fish expresses a GFP and a BFP.

32. (Previously presented) The method of claim 15, wherein the more than one fluorescent proteins are separately expressed in different tissues.

33. – 34. (Cancelled)

35. (Original) The method of claim 32, wherein the transgenic fish expresses a YFP under the control of a muscle specific promoter.

36. (Currently amended) The method of claim 1 or claim 43 ~~or 15~~, wherein the transgenic fish is a stable transgenic fish line obtained by a method comprising the steps of:

(a) obtaining a transgenic fish comprising one or more fluorescence genes positioned under the control of a promoter, wherein the transgenic fish expresses one or more fluorescent proteins encoded by the one or more fluorescence genes; and

(b) breeding the transgenic fish with a second fish to obtain offspring; and

(c) selecting from said offspring a stable transgenic line that expresses one or more fluorescent proteins.

37. (Original) The method of claim 36, wherein the second fish is a wild type fish.

38. (Original) The method of claim 36, wherein the second fish is a second transgenic fish.

39. (Currently amended) The method of claim 1 or claim 43 ~~or 15~~, wherein the transgenic fish is a transgenic zebrafish, medaka, goldfish or carp.

40. (Original) The method of claim 36, wherein the second fish is a zebrafish, medaka, goldfish or carp.

41. (Currently amended) The method of ~~claim 1 or 36~~claim 1, 36 or 43, wherein the transgenic fish is a transgenic koi, loach, tilapia, glassfish, catfish, angel fish, discus, eel, tetra, goby, gourami, guppy, Xiphophorus, hatchet fish, Molly fish, or pangasius.

42. (Previously presented) The method of claim 39, wherein the transgenic fish is a transgenic zebrafish.

43. (New) A method of providing transgenic fish to the ornamental fish market, comprising the steps of:

(a) obtaining a transgenic fish line comprising one or more chimeric genes that are positioned under the control of a promoter that drives the expression of a fluorescent protein in muscles of said fish, said promoter being a muscle specific promoter, such that said transgenic fish expresses fluorescent protein encoded by the gene in skeletal muscle

at a level sufficient such that said transgenic fish fluoresces upon exposure to one or more light; and

(b) distributing fish of said line to the ornamental fish market.

44. (New) The method of claim 1 or 43, wherein the transgenic fish express a GFP.

45. (New) The method of claim 44, wherein the transgenic fish express an EGFP.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/913,898	10/03/2001	Zhiyuan Gong	1781-0163P	5940

2292 7590 07/30/2003

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EXAMINER
WOITACH, JOSEPH T

ART UNIT	PAPER NUMBER
1632	13

DATE MAILED: 07/30/2003

Please find below and/or attached an Office communication concerning this application or proceeding.

File

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SERIAL NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NO.
09/913,898		Gong et al.	

EXAMINER	
Woitach, Joseph	
ART UNIT	**PAPER NUMBER**
1632	13

DATE MAILED:

Please find below a communication from the EXAMINER in charge of this application.
Commissioner of Patents

See Attached.

DETAILED ACTION

This application is a 371 national stage filing of PCT/SG99/00079, filed July 16, 1999 which claims benefit to foreign application 9900811-2, filed February 18, 1999 in Singapore.

Applicant's amendment filed May 12, 2003, paper number 12, has been received and entered. Claims 1-18 have been canceled. Claims 19-59 have been entered. Claims 19-59 are pending.

Election/Restriction

Applicants' response to the restriction requirement is noted. Specifically, Applicants note that claims 1-18 have been canceled, and that newly added claims 19-59 are 'directed to in essence a"method of doing business"' (page 6, Section II). Applicants indicate that the newly added claims are not believed to "fit into any of the restriction groups identified by the Examiner with respect to the previous claims" (page 6, Section II).

The amendment filed on May 12, 2003, paper number 12, canceling all claims drawn to the inventions set forth in the restriction requirement and presenting only claims drawn to a new invention is **non-responsive** (MPEP § 821.03). As acknowledged by Applicants, the remaining claims are not readable on the elected invention.

Since the above-mentioned amendment appears to be a bona fide attempt to reply, applicant is given a TIME PERIOD of ONE (1) MONTH or THIRTY (30) DAYS, whichever is longer, from the mailing date of this notice within which to supply the omission or correction in order to avoid abandonment. EXTENSIONS OF THIS TIME PERIOD UNDER 37 CFR 1.136(a) ARE AVAILABLE.

The original restriction requirement is re-iterated below for Applicants' convenience.

Restriction is required under 35 U.S.C. 121 and 372.

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1.

In accordance with 37 CFR 1.499, applicant is required, in reply to this action, to elect a single invention to which the claims must be restricted.

I. Claims 1, 5, 6, 14, 15, drawn to a zebrafish cytokeratin gene promoter, classified in class 536, subclass 24.1.

II. Claims 2, 5, 6, 14, 15, drawn to a zebrafish creatine kinase gene promoter, classified in class 536, subclass 24.1.

III. Claims 3, 5, 6, 14, 15, drawn to a zebrafish fast muscle isoform of myosin light chain 2 gene promoter, classified in class 536, subclass 24.1.

IV. Claims 4, 5, 6, 14, 15, drawn to a zebrafish ribosomal protein P0 gene promoter, classified in class 536, subclass 24.1.

V. Claims 7-13, 16, drawn a transgenic fish comprising a zebrafish cytokeratin gene promoter, classified in class 800, subclass 20.

VI. Claims 7-13, 16, drawn a transgenic fish comprising a zebrafish creatine kinase gene promoter, classified in class 200, subclass 20.

VII. Claims 7-13, 16, drawn a transgenic fish comprising a zebrafish fast muscle isoform of myosin light chain 2 gene promoter, classified in class 800, subclass 20.

VIII. Claims 7-13, 16, drawn a transgenic fish comprising a zebrafish ribosomal protein P0 gene promoter, classified in class 800, subclass 20.

IX. Claim 17, drawn a transgenic fish comprising a zebrafish ribosomal protein P0 gene promoter, classified in class 800, subclass 20.

X. Claim 18, drawn a transgenic fish comprising a zebrafish ribosomal protein P0 gene promoter, classified in class 800, subclass 20.

Claims 5, 6, 14, 15 are generic to groups I-IV and will be examined to the extent they encompass the elected invention. Claims 7-13, 16 are generic to groups V-VIII and will be examined to the extent they encompass the elected invention.

The inventions listed as Groups I-X do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

A) The invention has no special technical feature that defined the contribution over the prior art, or

B) Unity of invention between different categories of inventions will only be found to exist if specific combinations of inventions are present. Those combinations include:

1) A product and a special process of manufacture of said product.

2) A product and a process of use of said product.

3) A product, a special process of manufacture of said product, and a process of use of said product.

4) A process and an apparatus specially designed to carry out said process.

5) A product, a special process of manufacture of said product, and an apparatus specially designed to carry out said process.

The allowed combinations do not include multiple products, multiple methods of using said products, and methods of making multiple products as claimed in the instant application, see MPEP § 1850. In the instant case, Applicant's claims encompass multiple inventions and do not have a special technical feature which link the inventions one to the other, and lack unity of invention.

Specifically, the inventions are distinct, each from the other because of the following reasons:

Inventions I-IV are unrelated. Inventions are unrelated if it can be shown that they are not disclosed as capable of use together and they have different modes of operation, different functions, or different effects (MPEP § 806.04, MPEP § 808.01). In the instant case the different polynucleotide sequences represent unique and different promoter sequences with different inherent properties as demonstrated by a particular expression pattern in a cell.

Inventions V-VIII are unrelated. Inventions are unrelated if it can be shown that they are not disclosed as capable of use together and they have different modes of operation, different functions, or different effects (MPEP § 806.04, MPEP § 808.01). In the instant case each of the transgenic fish comprise a different promoter which results in a material different genome in the transgenic fish. Additional, the inherent activity of each of the promoters if expressed may provide a unique phenotype to each of the transgenic fish of each of the inventions.

Inventions I-IV and V-VIII, respectively, are related as product and process of use. The inventions can be shown to be distinct if either or both of the following can be shown: (1) the process for using the product as claimed can be practiced with another materially different product or (2) the product as claimed can be used in a materially different process of using that product (MPEP § 806.05(h)). In the instant case the promoters can be used for expression studies in vitro, as probes for detecting the presence of the respective gene, or as a target sequence for an anti-sense oligonucleotide.

Inventions IX and X are unrelated. Inventions are unrelated if it can be shown that they are not disclosed as capable of use together and they have different modes of operation, different functions, or different effects (MPEP § 806.04, MPEP § 808.01). In the instant case the methods require a transgenic fish comprising different promoters. The genome of the transgenic fish in each case are different and unique thus the methods require different starting materials. Further, the methods require the administration and analysis of two different classes of compounds; steroid-like compounds and heavy metals.

Inventions V-VIII and IX-X are unrelated. Inventions are unrelated if it can be shown that they are not disclosed as capable of use together and they have different modes of operation, different functions, or different effects (MPEP § 806.04, MPEP § 808.01). In the instant case the methods require a transgenic fish comprising promoters which are different from those specifically set forth in the inventions of groups V-VIII.

Because these inventions are distinct for the reasons given above and have acquired a separate status in the art because of their recognized divergent subject matter, restriction for examination purposes as indicated is proper. Further, because these inventions are distinct for the reasons given above and the search required for each on of Group I-X is not required or coextensive with each other, restriction for examination purposes as indicated is proper.

Applicant is advised that the reply to this requirement to be complete must include an election of the invention to be examined even though the requirement be traversed (37 CFR 1.143).

Applicant is reminded that upon the cancellation of claims to a non-elected invention, the inventorship must be amended in compliance with 37 CFR 1.48(b) if one or more of the currently named inventors is no longer an inventor of at least one claim remaining in the application. Any amendment of inventorship must be accompanied by a request under 37 CFR 1.48(b) and by the fee required under 37 CFR 1.17(i).

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Joseph Woitach whose telephone number is (703)305-3732.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Deborah Reynolds, can be reached at (703)305-4051.

Any inquiry of a general nature or relating to the status of this application should be directed to the Group analyst Dianiece Jacobs whose telephone number is (703) 308-2141.

Joseph T. Woitach

Deborah Crouch

DEBORAH CROUCH
PRIMARY EXAMINER
GROUP 1800/1630

X. RELATED PROCEEDINGS APPENDIX

There are no other appeals, interferences, or judicial proceedings which will directly affect or be directly affected by or have a bearing on the Board's decision in this appeal.